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THE UNIVERSITY OF ALBERTA

PROPERTIES OF LARGE α TROPOMYOSIN FRAGMENTS PREPARED BY SELECTIVE ENZYMIC AND CHEMICAL CLEAVAGES

by

© MARY DENCE PATO

A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
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FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled "Properties of Large α Tropomyosin Fragments Prepared by Selective Enzymic and Chemical Cleavages" submitted by Mary Dence Pato in partial fulfilment of the requirements for the Degree of Doctor of Philosophy.

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ABSTRACT

Several large fragments of rabbit skeletal αTm have been prepared by selective enzymic and chemical methods. Limited tryptic digestion resulted in the hydrolysis of a single susceptible bond, 133-134, producing T1 (1-133) and T2 (134-284). Prolonged exposure to the enzyme formed trypsin resistant fragments, T3 (13-125), T4 (183-284) and T5 (183-244). Limited chymotryptic digestion selectively hydrolyzed peptide bond 169-170 producing CT1 (1-169) and CT2 (170-284). Non-polymerizable Tm was prepared by treatment with carboxypeptidase A. The combined action of DTNB and KCN cleaved peptide bond 189-190 producing Cy1 (1-189) and Cy2 (190-284) while CN1A (11-127) and CN1B (142-281) were obtained from digestion of αTm with CNBr. Using the modes of action of trypsin and chymotrypsin as a probe for regions of less ordered structure, it was observed that the N-terminus (residues 1-12) and the central part of the molecule (residues 126-182) are less likely to be helical than the remainder of αTm. Circular dichroism studies showed that the NH_2-terminal fragments have higher helical content and greater thermal stability than fragments from the COOH-terminal half of the molecule. The COOH-terminal fragments and non-polymerizable Tm bind with a significantly higher affinity to TnT immobilized on Sepharose 4B than the NH_2-terminal fragments but less tightly than intact αTm. Mixtures of fragments capable of head to tail aggregation

indicated complex formation and exhibited better binding affinities than the individual components. The results of the binding studies of Tm and its fragments with Tn and its components are compatible with two possibilities for the location of Tn interaction site on Tm. It can be located either at one-third of the distance from the C-terminus of the molecule or at the end to end overlap. In either case, the formation of a head-to-tail complex enhances the affinity for TnT binding and possibly vice versa. None of the fragments except the carboxypeptidase treated Tm bind to F-actin.

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LIST OF ABBREVIATIONS

AH	aminohexyl
ATP	adenosine triphosphate
BSA	bovine serum albumin
C	carboxyl
CB1	TnT fragment, residues 1-151
CH	carboxyhexyl
CM	carboxymethyl
CNBr	cyanogen bromide
CN1A	αTm fragment, residues 11-127
CN1B	αTm fragment, residues 142-281
COOH	carboxyl
CT1	αTm fragment, residues 1-169
CT2	αTm fragment, residues 170-284
Cy1	αTm fragment, residues 1-184
Cy2	αTm fragment, residues 190-284
DTT	dithiotreitol
DFP	diisopropylphosphofluoridate
DTNB	5,5' dithiobis (2-nitrobenzoic acid)
$E^{1\%}_{\lambda,\ 1\ \mathrm{cm}}$	absorbance of a 1% protein solution in a 1 cm pathlength cell at the wavelength, λ
EGTA	ethyleneglycol-bis-(β-aminoethyl ether) N,N'-tetraacetic acid
EDC	1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride
LMM	light meromyosin
Nbs_2	5,5' dithiobis (2-nitrobenzoic acid)
N	amino

NH_2	amino
PAGE	polyacrylamide gel electrophoresis
$P\alpha$	α helical conformational parameter
$P\beta$	β sheet conformational parameter
Pr.c.	random coil conformational parameter
PTH	phenylthiohydrantoin
QAE	quarternary aminoethyl
$S_{20,w}$	sedimentation coefficient corrected to water at 20°C
SDS	sodium dodecyl sulfate
TCA	trichloroacetic acid
Tm	tropomyosin
T1	αTm fragment, residues 1-133
T2	αTm fragment, residues 134-284
T3	αTm fragment, residues 13-125
T4	αTm fragment, residues 183-284
T5	αTm fragment, residues 183-244
Tn	troponin complex
TnC	the calcium binding protein of the troponin complex
TnI	the ATPase inhibitory protein of the troponin complex
TnT	the tropomyosin binding component of the troponin complex
TnCT	complex of troponin C and troponin T
TPCK	L-1-tosylamido-2-phenylethyl chloromethyl ketone
Tris	tris-(hydroxymethyl) aminomethane
$T_{1/2}$	melting temperature
λ	wavelength

$[\theta]_\lambda$	mean residue ellipticity at the wavelength, λ
θ_{obs}	observed ellipticity value

CHAPTER I

INTRODUCTION

A. VERTEBRATE STRIATED MUSCLE

The transformation of the chemical energy of ATP into mechanical energy of contractile processes is one of the most intriguing problems in biochemistry. Thus, a great deal of research has been directed towards the elucidation of the mechanism of contraction. Models for the mechanism and regulation of this process in striated muscle have been formulated from the numerous experimental observations obtained from work done in this system. A brief review of the structure of muscle and these models will be presented in this chapter. This area is covered by several excellent reviews written on the subject (Mannherz & Goody, 1976; Squire, 1975 and Weber & Murray, 1973).

Vertebrate muscle, under voluntary control, has a striated appearance when viewed under the light microscope (Fig. 1). The characteristic striation is due to alternating optically dense (A band) and less dense (I band) transverse regions along the myofibrils which compose the muscle fiber (Fig. 2). Under polarized light, A band is strongly birefringent while the I band is relatively non-birefringent.

The Z lines bisect the I band and the space between

Fig. 1. Electron micrograph of a longitudinal section of rabbit psoas muscle.
[By H.E. Huxley cited in Perry, 1960].

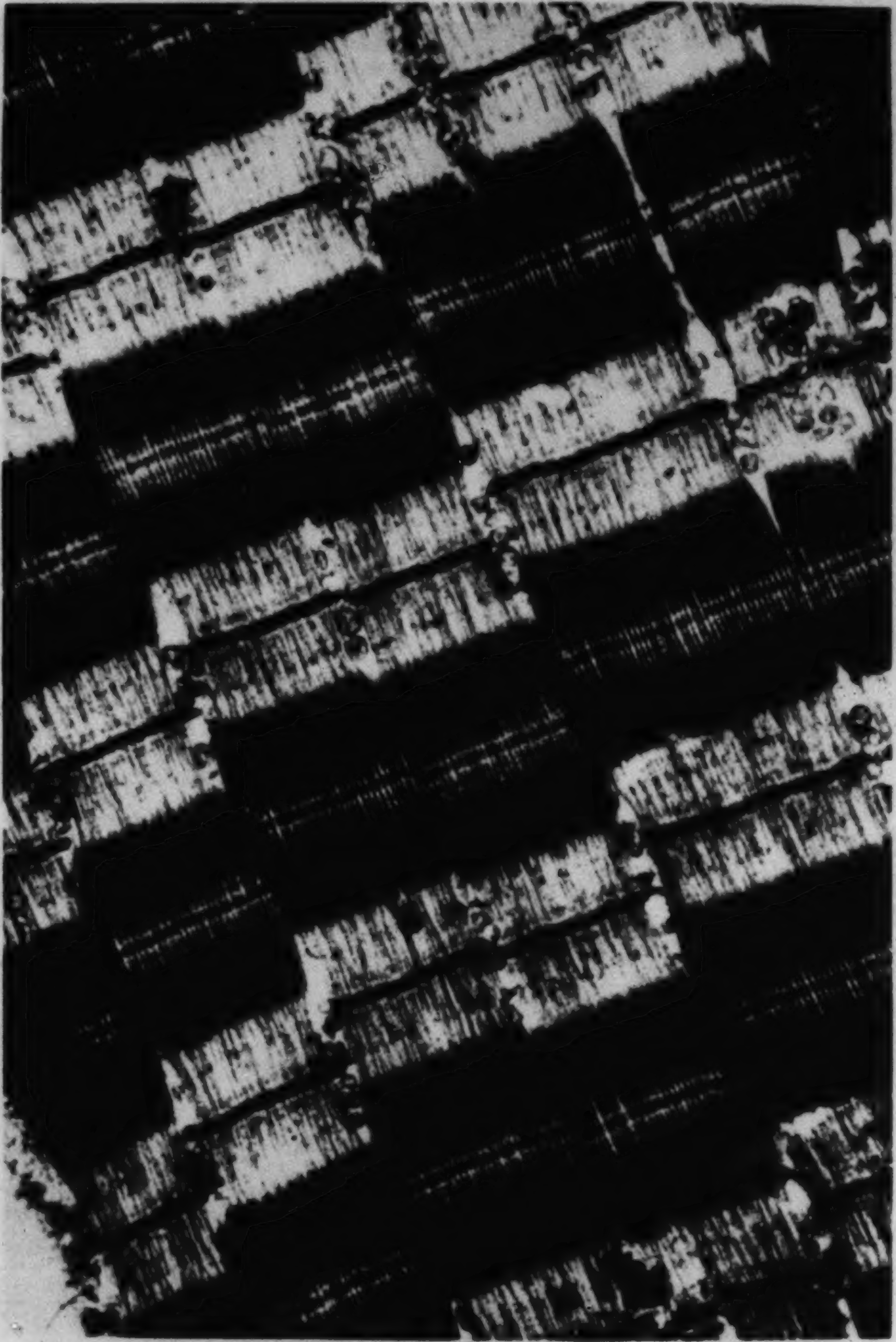


Plate A

Electron micrograph of a longitudinal section of rabbit psoas muscle (by H. E. Huxley cited in Perry, 1960).

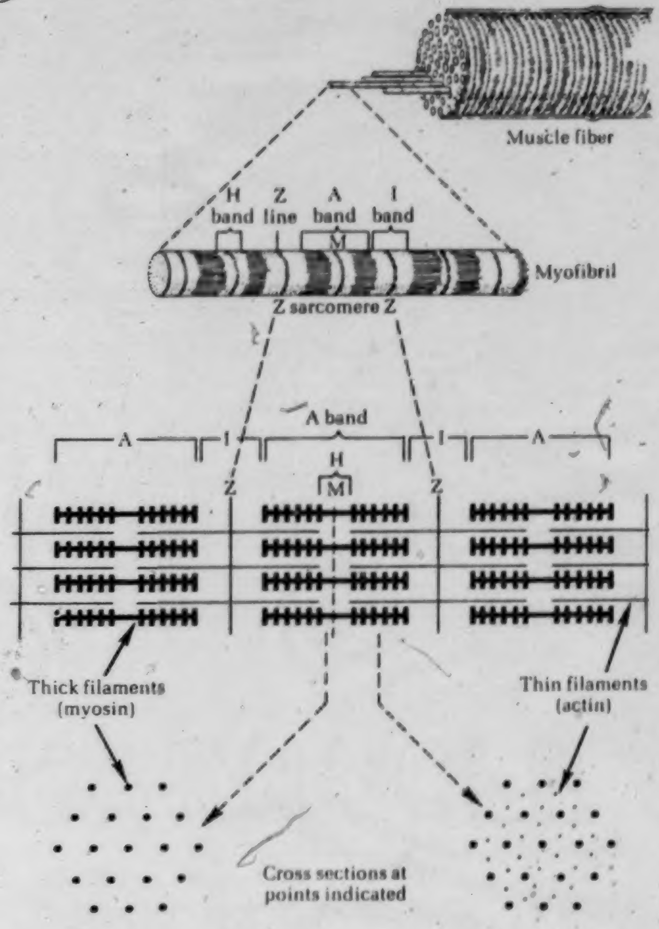

Muscle fiber
H band
Z line
A band
I band
M
Myofibril
Z sarcomere Z
A band
A
I
H
M
I
A
Z
Z
Thick filaments (myosin)
Thin filaments (actin)
Cross sections at points indicated

Fig. 2. Schematic diagram of the structure of the myofibril [From Lehninger, 1970].

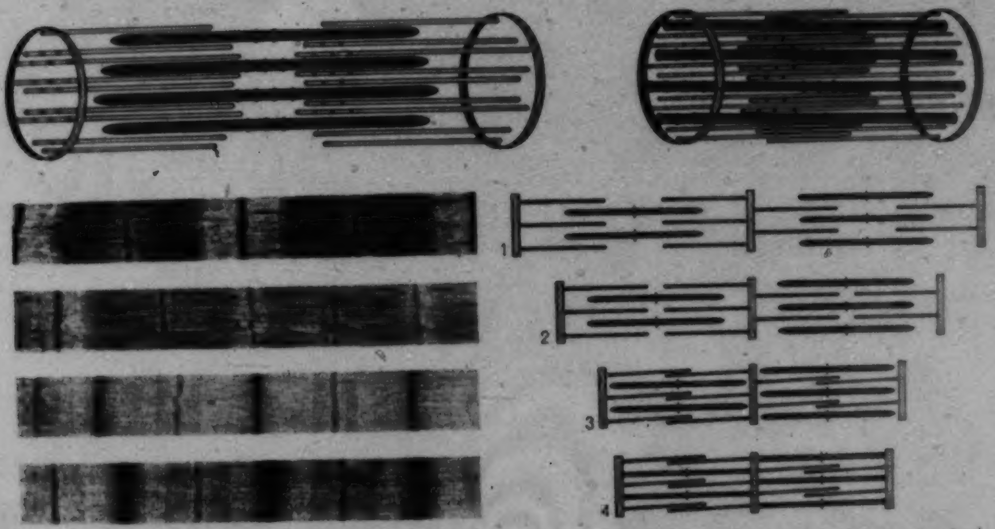
two adjacent Z lines defines the sarcomere, the basic contractile unit. It is composed of a remarkable array of protein myofilaments, the thin and thick filaments.

The thick filaments are about 140 Å in diameter, about 1.6 μm long and occupy the central part of the sarcomere. They are made up of myosin molecules aggregated in a tail to tail manner resulting in the formation of a bare smooth region at the middle of the filament and projections of the myosin heads at the ends in opposite directions. Adjacent myosin filaments are joined halfway along their length by a crosslinking structure called the M-line.

Interdigitating with the array of myosin filaments are two sets of thin filaments which are about 1 μm long and about 70-80 Å in diameter. Arrays of thin filaments on one edge of the sarcomere are linked to a similar array in the adjacent sarcomere by the Z-line. Under certain conditions, electron microscopy has detected the formation of interfilamentous crossbridges between the two filament types in the region where the thick and thin filaments overlap.

Contraction is signalled by an impulse which causes the release of Ca^{++} from the sarcoplasmic reticulum into the sarcoplasm raising its Ca^{++} concentration to about 10^{-5}M. The sarcomere shortens but the lengths of the myofilaments do not change appreciably. These observations led to the sliding filament model of muscle contraction illustrated in Fig. 3 (Huxley & Hanson, 1954, 1960). The model entails

Fig. 3. Four consecutive stages of muscle contraction are shown by the electron micrographs and accompanying schematic illustrations (1,2,3,4) of muscle in longitudinal sections. The sliding filament theory can explain the shortening of the sarcomere length while maintaining the length of the myofibrils (top left and right). [From Huxley, 1965].



CONTRACTION OF MUSCLE entails change in relative position of the thick and thin filaments that comprise the myofibril (*top left and right*). The effect of contraction on the band pattern of muscle is indicated by four electron micrographs and accompanying schematic illustrations of muscle in longitudinal section, fixed at consecutive stages of contraction. First the *H*-zone closes (*1*), then a new dense zone develops in the center of the *A* band (*2, 3 and 4*) as thin filaments from each end of the sarcomere overlap.

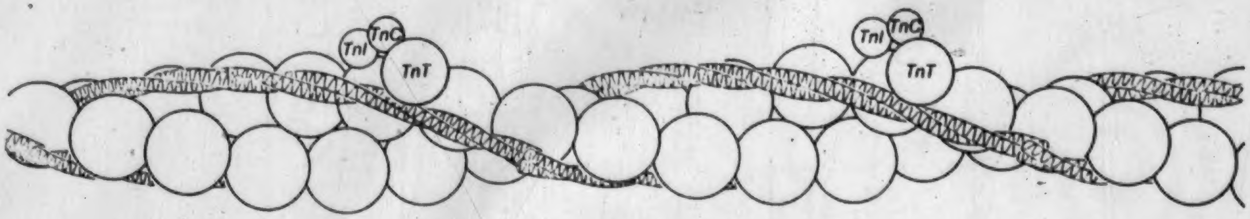
the relative sliding motion of the filaments past each other as a result of constant formation and disruption of the cross bridges during contraction. The energy required for this mechanical motion is provided by the energy released from the hydrolysis of ATP which accompanies contraction.

A better understanding of this process and its regulation was achieved from the isolation and study of the biochemical properties of the pure forms of the major proteins of the myofilaments.

Myosin molecules, which make up the thick filament, consist of two heavy chains and four light chains, with a total molecular weight of about 470,000. The two heavy chains associate to form a long α helical rod with two globular heads at one end, with which the light chains are associated. Myosin hydrolyzes ATP and this function is localized at the globular heads. Dissociation of a pair of light chains, known as alkaline light chains, results in loss of activity.

On the other hand, the thin filament is composed of three proteins, actin, troponin (Tn) and tropomyosin (Tm) existing in a 7:1:1 molar ratio. The structural arrangement of these proteins in the thin filament is illustrated in the model proposed by Ebashi, et al. (1969) (Fig. 4).

The major protein component of the thin filament is G-actin, a globular protein of molecular weight of 42,000. Its primary sequence has been determined (Collins & Elzinga,



TnI
TnC
TnT
TnI
TnC
TnT

Fig. 4. Model for the structural organization of the thin filament proteins of striated muscle. [From Cohen, 1975].

1975). In the presence of salt and ATP, G-actin molecules polymerize into an F-actin filament, often described as a string of pearls. The thin filament is made up of two F-actin strands wound around each other. Along each of the grooves of the F-actin filament lies an end to end polymer of rod-shaped tropomyosin molecules. Each tropomyosin monomer traverses 7 actin monomers of one strand and is associated with a troponin molecule at a specific site. Tn is composed of three subunits, troponin I (TnI), troponin C (TnC) and troponin T (TnT). TnI is a basic protein with a molecular weight of about 21,000 and known primary sequence (Wilkinson & Grand, 1975). It inhibits the actin myosin interaction in conjunction with Tm. This inhibitory effect is reversed by TnC, an acidic protein with a molecular weight of 18,000, which undergoes a conformational change as it binds Ca^{++}. Its complete sequence has already been determined (Collins, 1974). The Tn complex associates with Tm through the TnT component. TnT is a globular, basic protein of molecular weight of about 31,000. Analysis of the complete sequence (Pearlstone, et al., 1976) revealed that it has α helical regions and about 80% of the helical content of the molecule is localized at residues 71-151 (Pearlstone & Smillie, 1977). This particular region was also found to be the Tm interaction site on TnT (Jackson, et al., 1975).

In cross-section, the thin filament proteins in resting muscle may be visualized as in Fig. 5a. The Tn

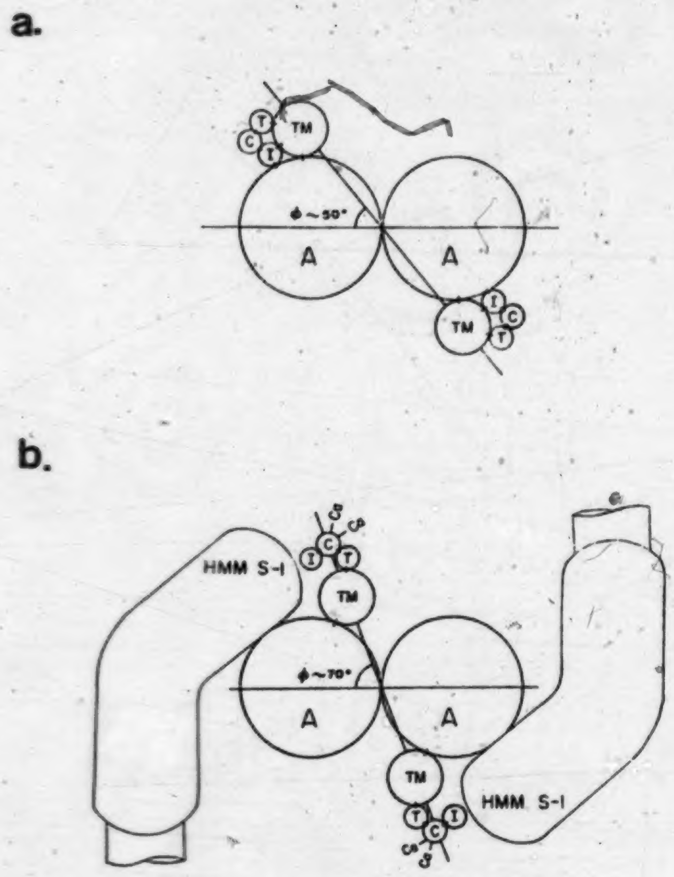

a.
TM
T
C
I
ϕ ~ 50°
A
A
TM
I
C
T
b.
Ca
Ca
C
I
T
HMM S-I
TM
ϕ ~ 70°
A
A
TM
T
C
I
Ca
HMM S-I

Fig. 5. Model for regulation of muscle contraction. Schematic diagrams of the cross-section of the thin filament in the relaxed state (pCa ≈ 8) [a] and contracted state (pCa ≈ 5) [b] show the movement of tropomyosin (Tm) and troponin subunits (T,C,I) at high Ca^{++} concentration allowing interaction between the heavy meromyosin subfragment one position. [From Potter & Gergely, 1974].

complex is bound to Tm through TnT and to a region made up of Tm and actin through TnI. In this configuration the tropomyosin-troponin complex sterically blocks the myosin binding site on actin. Upon the arrival of the nerve impulse, the intracellular Ca^{++} concentration increases from $10^{-7}M$ to about $10^{-5}M$. TnC binds Ca^{++} and undergoes a conformational change which is transmitted to Tm through TnT. The Tm filament then moves deeper into the grooves of the actin filaments (Fig. 5b) exposing the interaction site on actin to myosin heads allowing cross bridges to form. ATP is hydrolyzed and contraction occurs. At the end of the impulse, Ca^{++} is sequestered and Tm filament rolls back to its resting position.

B. REGULATION OF MUSCLE CONTRACTION

Thus, the control of contractile activity is achieved primarily by regulating the interaction of actin and myosin heads and consequently the rate of hydrolysis of ATP by the regulatory proteins, Tn and Tm, which is ultimately controlled by the Ca^{++} concentration of the sarcoplasm. In all types of muscle, so far investigated, the intracellular concentration of Ca^{++} must rise to about $10^{-5}M$ for ATP hydrolysis to proceed at a significant rate and for contraction to occur.

A less obvious role of Ca^{++} in the regulation of muscle contraction is its involvement in phosphorylation of contractile proteins. For several years now, this phenomenon has been recognized but it is only recently that its significance has become clearer. (For review see Perry, 1976 and Adelstein, 1978.) Among the myofibrillar proteins that are phosphorylated are muscle (skeletal, cardiac, smooth) and non-muscle (platelet, proliferative myoblast and astrocyte) myosin P-light chains, skeletal TnT, TnI and αTm. The requirement of phosphorylation of myosin P-light chains for actin activated myosin ATPase in non-muscle cells has been demonstrated by Scordilis and Adelstein (1977) and in chicken gizzard by Gorecka, et al., (1976) and Sobieszek (1977). Phosphorylation of these polypeptides is mediated by myosin light chain kinase which requires Ca^{++} for activity. However, the importance of phosphorylation of skeletal contractile proteins is not fully understood yet since it does not appear to affect the extent of actin activated myosin ATPase appreciably.

C. TROPOMYOSIN

A full understanding of the Tm-Tn regulatory mechanism requires detailed studies of the nature and specificity of the interactions between the contractile proteins. To this end, Tm, the protein which links Tn and actin, has been well characterized physically and chemically. (For review see Smillie, 1976.)

Rabbit skeletal Tm exhibits a molecular weight of 66,000 at high salt concentration and dissociates into monomers of about 33,000 in the presence of denaturants under reducing conditions. The ratios of the two components of Tm from various sources differ and in rabbit skeletal Tm, the ratio of the α to the β component is 3.5:1. The occurrence of αα and αβ dimers but not ββ dimers has been demonstrated (Yamaguchi, et al., 1974 and Lehrer, 1975). Chromatography of Tm on CM-32 cellulose at pH 4.0 in the presence of 8M urea separates the α from the β component (Cummins & Perry, 1973).

Tm has a rod like shape and very high α helical content (Cohen & Szent-Gyorgyi, 1957) qualifying it as the simplest and smallest member of the k-m-e-f groups of fibrous proteins. Crick (1953) had proposed that the main feature of the X-ray diffraction pattern of the α group of fibrous proteins were explicable in terms of coiled-coil structure in which 2 or 3 right handed helices, running in the same direction, are wrapped around each other. This model entails the occurrence of a regular pattern of non-polar amino acids every 3.5 residues packed in a knob-into-hole manner to form the hydrophobic core while the polar residues will be situated in the outside of the molecule.

Determination of the primary structure of the α chain, major component of rabbit skeletal Tm, confirmed Crick's hypothesis (Stone, et al., 1974 and Hodges, et al., 1972). The complete sequence of αTm (Fig. 6) shows the occurrence of two series of non-polar residues at an interval of seven amino acids throughout the entire length of the

Fig. 6. Amino acid sequence of α tropomyosin. Nonpolar residues occur in two series, I (squared) and II (circled).
[From Stone, _et al_., 1974].

	1 AcMet	2 Asp	3 Ala	4 Ile	5 Lys	6 Lys	7 Lys	8 Met	9 Gln	10 Met	11 Leu	12 Lys	13 Leu
14 Asp	15 Lys	16 Glu	17 Asn	18 Ala	19 Leu	20 Asp	21 Arg	22 Ala	23 Glu	24 Gln	25 Ala	26 Glu	27 Ala
28 Asp	29 Lys	30 Lys	31 Ala	32 Ala	33 Glu	34 Asp	35 Arg	36 Ser	37 Lys	38 Gln	39 Leu	40 Glu	41 Asp
42 Glu	43 Leu	44 Val	45 Ser	46 Leu	47 Gln	48 Lys	49 Lys	50 Leu	51 Lys	52 Gly	53 Thr	54 Glu	55 Asp
56 Glu	57 Leu	58 Asp	59 Lys	60 Tyr	61 Ser	62 Glu	63 Ala	64 Leu	65 Lys	66 Asp	67 Ala	68 Gln	69 Glu
70 Lys	71 Leu	72 Glu	73 Leu	74 Ala	75 Glu	76 Lys	77 Lys	78 Ala	79 Thr	80 Asp	81 Ala	82 Glu	83 Ala
84 Asp	85 Val	86 Ala	87 Ser	88 Leu	89 Asn	90 Arg	91 Arg	92 Ile	93 Gln	94 Leu	95 Val	96 Glu	97 Glu
98 Glu	99 Leu	100 Asp	101 Arg	102 Ala	103 Gln	104 Glu	105 Arg	106 Leu	107 Ala	108 Thr	109 Ala	110 Leu	111 Gln
112 Lys	113 Leu	114 Glu	115 Glu	116 Ala	117 Glu	118 Lys	119 Ala	120 Ala	121 Asp	122 Glu	123 Ser	124 Glu	125 Arg
126 Gly	127 Met	128 Lys	129 Val	130 Ile	131 Glu	132 Ser	133 Arg	134 Ala	135 Gln	136 Lys	137 Asp	138 Glu	139 Glu
140 Lys	141 Met	142 Glu	143 Ile	144 Gln	145 Glu	146 Ile	147 Gln	148 Leu	149 Lys	150 Glu	151 Ala	152 Lys	153 His
154 Ile	155 Ala	156 Glu	157 Asp	158 Ala	159 Asp	160 Arg	161 Lys	162 Tyr	163 Glu	164 Glu	165 Val	166 Ala	167 Arg
168 Lys	169 Leu	170 Val	171 Ile	172 Ile	173 Glu	174 Ser	175 Asp	176 Leu	177 Glu	178 Arg	179 Ala	180 Glu	181 Glu
182 Arg	183 Ala	184 Glu	185 Leu	186 Ser	187 Glu	188 Gly	189 Lys	190 Cys	191 Ala	192 Glu	193 Leu	194 Glu	195 Glu
196 Glu	197 Leu	198 Lys	199 Thr	200 Val	201 Thr	202 Asn	203 Asn	204 Leu	205 Lys	206 Ser	207 Leu	208 Glu	209 Ala
210 Gln	211 Ala	212 Glu	213 Lys	214 Tyr	215 Ser	216 Gln	217 Lys	218 Glu	219 Asp	220 Lys	221 Tyr	222 Glu	223 Glu
224 Glu	225 Ile	226 Lys	227 Val	228 Leu	229 Ser	230 Asp	231 Lys	232 Leu	233 Lys	234 Glu	235 Ala	236 Glu	237 Thr
238 Arg	239 Ala	240 Glu	241 Phe	242 Ala	243 Glu	244 Arg	245 Ser	246 Val	247 Thr	248 Lys	249 Leu	250 Glu	251 Lys
252 Ser	253 Ile	254 Asp	255 Asp	256 Leu	257 Glu	258 Asp	259 Glu	260 Leu	261 Tyr	262 Ala	263 Gln	264 Lys	265 Leu
266 Lys	267 Tyr	268 Lys	269 Ala	270 Ile	271 Ser	272 Glu	273 Glu	274 Leu	275 Asp	276 His	277 Ala	278 Leu	279 Asn
280 Asp	281 Met	282 Thr	283 Ser	284 Ile									

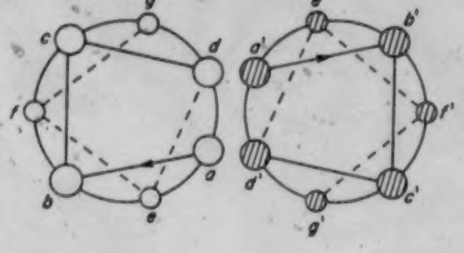
molecule (Stone, et al., 1974). Amino acids in series I positions are three residues on the NH_2-terminal side of and four residues on the COOH-terminal side of residues in series II. The pattern clearly provides, on the average, a non-polar amino acid at every 3.5 residues and is consistent with the coiled coil model.

At several positions in either series I or II, there occur amino acids which are not ordinarily classified as hydroprobes like Lys 15 and 29, Gln 144 and 263, Glu 218, Asp 137, Ser 36, 123 and 186, Thr 53, Cys 190 and Tyr 60, 162, 214, 221 and 267. However, the polar or non-polar nature of these residues, with the exception of Glu, Asp and Lys, is ambivalent. Model building studies (Hodges, et al., 1972) have shown that they can be accommodated in the coiled coil structure by directing their polar moiety towards the exterior except for Asp-137 because of its shorter side chain length. On the other hand it is possible that their occurrence at certain positions in the hydrophobic pattern leads to local disruption of the regularity of the coiled coil and may be important in the interaction of Tm with actin and Tn.

Molecular packing of the two α helical chains of Tm into a coiled coil structure would necessitate the pairing of hydrophobes of one series in one chain with amino acids of the same series in the other chain. Thus, the two chains can either be in axial register or staggered by seven residues or some multiple of seven residues. On the basis of model building considerations, McLachlan and Stewart (1975) have argued that the hydrophobic groups make a better fit when the two helices are in register. In agreement with these findings, direct experimental evidence has been reported from various laboratories (Johnson & Smillie, 1975, Lehrer, 1975, and Stewart, 1975). The unique cysteine residue of both chains of Tm can be oxidized to form inter-chain disulfide bond without an increase in molecular weight. The necessity for proximity of the sulphydryl groups for this cross-linkage to occur indicated that the chains are in register.

αTm, which has 284 amino acids, can be considered as a molecule of forty repeating heptapeptides, with a final short period of four residues. Each heptapeptide or period occupies almost two turns of standard α helix with 3.6 residues per turn. McLachlan & Stewart (1975) designated the seven positions of each period as a, b, c, d, e, f and g, where a and d correspond to positions occupied by amino acids in series I and II respectively. The spatial arrangement of these amino acids in the coiled coil structure (Fig. 7a) shows that the proximity of amino acids in a and d positions allows them to interact and form the hydrophobic core of the molecule. Although amino acids in e and g positions are not very close, interaction between them is still possible. Examination of the sequence revealed that there is spatial distribution of acidic and basic residues (Stone, et al., 1974 and Parry, 1974) and further statistical

a.



b.

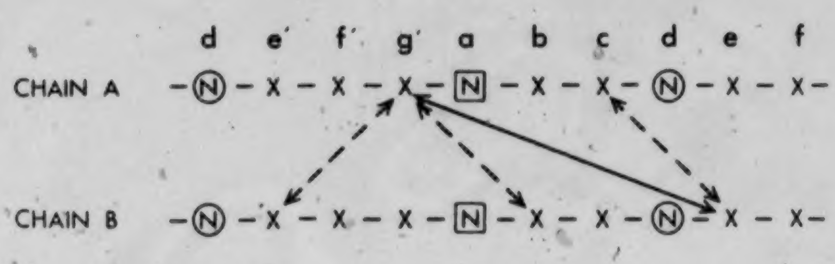

d e' f' g' a b c d e f
CHAIN A
–Ⓝ – X – X – X – N – X – X – Ⓝ – X – X–
CHAIN B
–Ⓝ – X – X – X – N – X – X – Ⓝ – X – X–

Fig. 7a. End-on view looking from amino to carboxyl end of two α helical chains of αTm in a coiled coil structure. The non-polar residues in the a and d positions (corresponding to series I and II positions) of one helix interlock with amino acids in the same positions of the other helix. [From McLachlan & Stewart, 1975].

Fig. 7b. Possible ionic interaction between heptapeptide sequences of two chains of αTm. The interactions between amino acid residues indicated by broken lines are limited by the presence of bulky hydrophobes other than alanine in the a and d positions of chain A unlike the interaction between g and e (indicated by solid line). [From Stone, et al., 1974].

analysis (McLachlan & Stewart, 1975) showed that a high proportion of amino acids in position e are acidic while many of those at g positions are basic. Thus ionic interaction of an amino acid in an e position in one helix could occur with the amino acid at the g position of the preceding heptapeptide of the second helix (Fig. 7b) and provide additional stabilization to the coiled coil structure. An antiparallel arrangement of the chains would result in the juxtapositioning of the amino acids of the same charges leading to destabilization of the structure.

D. ACTIN-TROPOMYOSIN INTERACTION

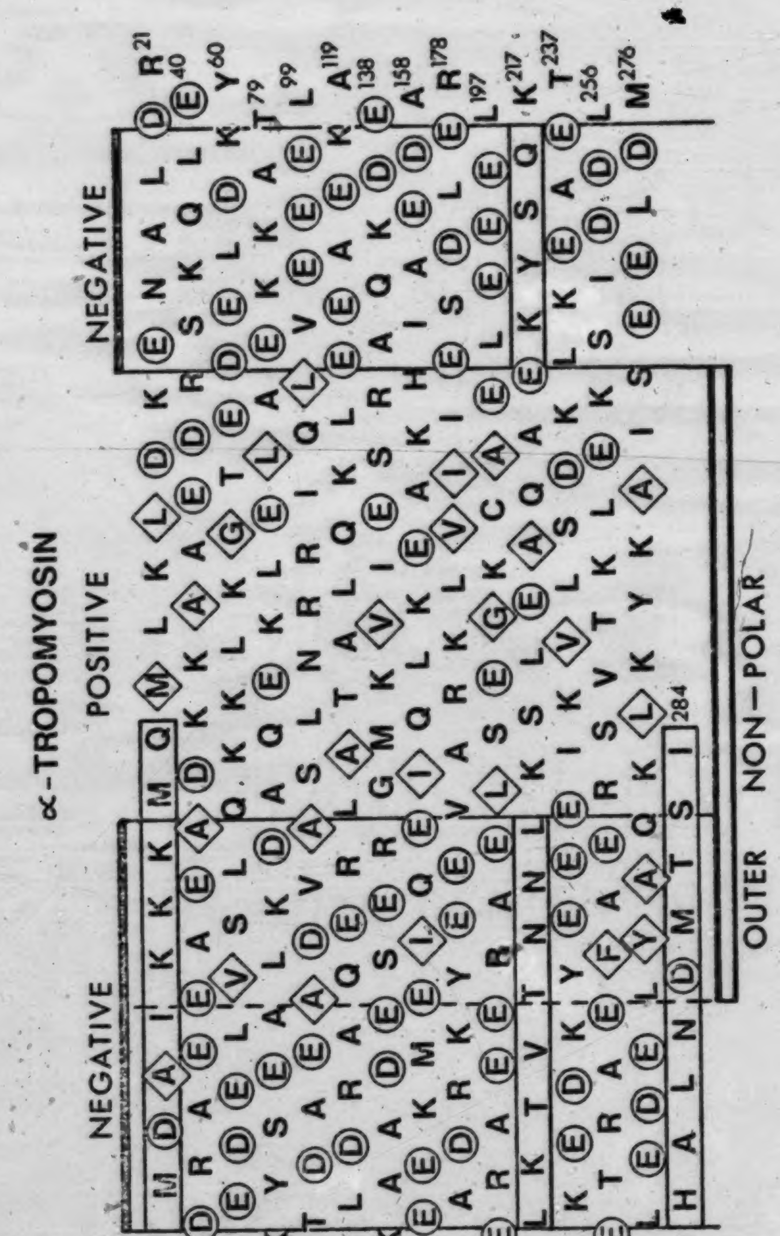
In the thin filament, Tn binds to Tm which in turn is bound to seven monomers on each of the two strands of F-actin. If each Tm molecule interacts with these seven actin monomers in an identical fashion, one might expect two sets of seven identical sequences repeated throughout the sequence corresponding to the binding sites of actin. No such identical repeating pattern was found upon inspection of the sequence (Stone, *et al.*, 1974). However, the possibility that similar or quasi-equivalent spatial arrangements of charged and non-polar groups on the surface of the coiled coil repeated along the entire length of the molecule was considered.

Analysis of paracrystals of Tm stained with uranyl acetate revealed a 395 Å periodicity as well as subperiods of 28 Å (395/14) (Parry & Squire, 1973). The fact that this distance is the same as the axial separation of subunits of F-actin suggested that these staining properties were due to a periodic distribution of non-polar and charged amino acids in the amino acid sequence of the molecule. Such a repeating pattern was detected by Parry (1974) and Miller (1975) from an inspection of the sequence of the COOH-terminal half of the molecule and confirmed for the whole molecule by Stone, et al., (1974). These authors pointed out the occurrence of a 19.5 residue periodicity involving acidic, basic and non-polar residues (excluding those in series I and II core positions) repeated fourteen-fold. Refined computational and statistical analysis (McLachlan & Stewart, 1976a) revised this periodicity to 19.66 and showed that the periodicity of the acidic and non-polar residues is significant while the basic residue distribution appears to be random.

The αTm sequence divides into 14 periods of 19 2/3 residues (Fig. 8). Each period comprises a narrow zone of net positive charge, a broader negative charged zone and a hydrophobic zone which overlaps every positive zone. Anomalies in the charge distribution occur at the ends of the molecule as well as near the unique cysteine residue (boxed regions of Figure 8). The former have been attributed to the end to end overlap sites upon head to tail aggregation of Tm molecules resulting in an apparent molecular length of 275 residues. This phenomenon allows the

Fig. 8. Amino acid sequence of αTm, in one letter code, drawn in 19.66 residue repeat pattern of 14 bands in the first 275 residues. Each band has a positive zone of 8 amino acids; a negative zone of 11 2/3 amino acids rich in acidic residues (o) and a hydrophobic zone rich in non-polar residues (◇) in the b, c and f positions.
[From McLachlan & Stewart, 1976a].

4


∝-TROPOMYOSIN
NEGATIVE
POSITIVE
NEGATIVE
OUTER
NON-POLAR
1
2
3
4
5
6
7
8
9
10
11
12
13
14
R21
E40
Y60
T79
L99
A119
138
A158
R178
L197
K217
T237
L256
M276
284

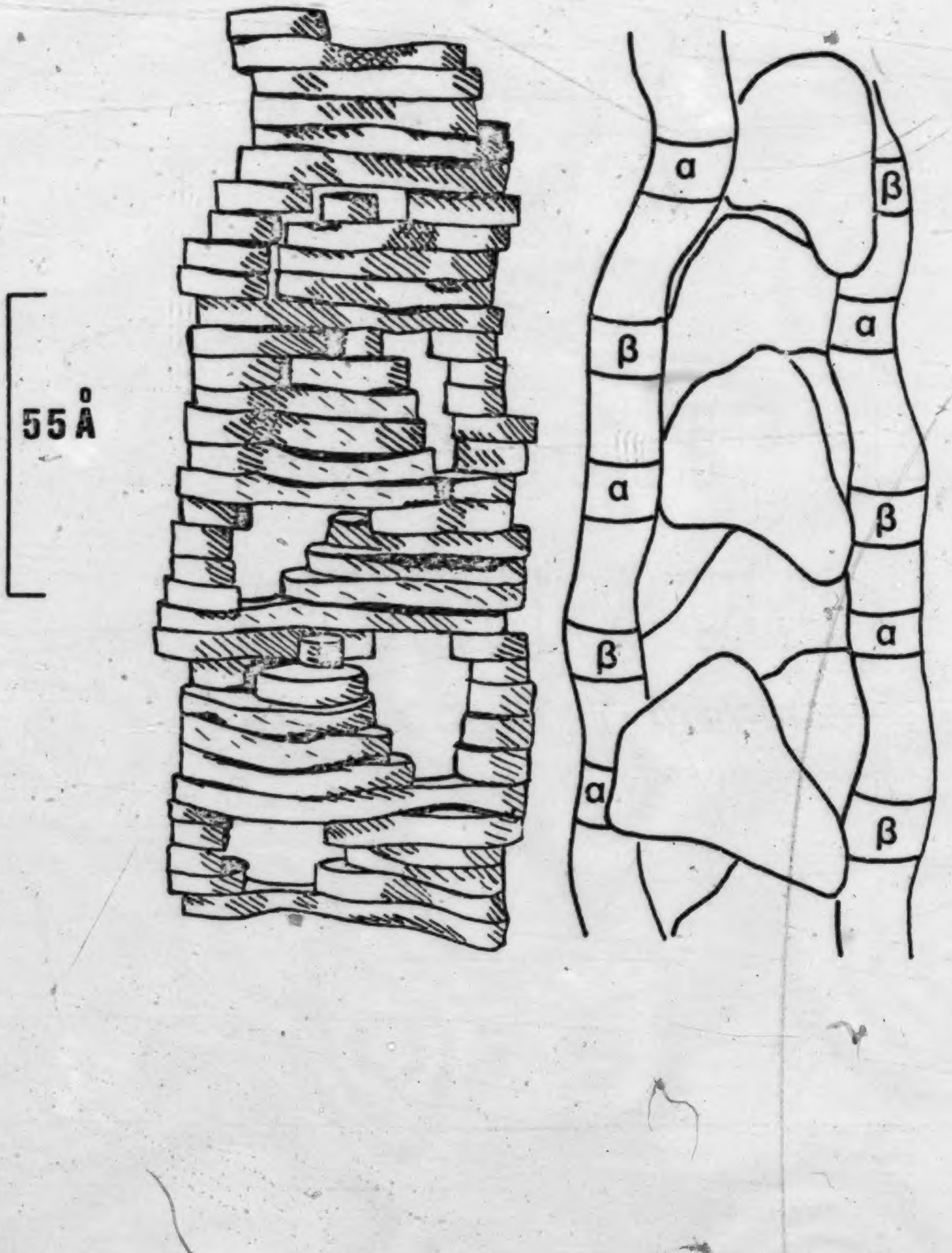
repeating pattern (14 x 19.66 = 275) to be carried over to the next molecule without interruption and would allow the presumptive pseudo equivalent actin binding sites of Tm to interact similarly with actin monomers over an extended length of the thin filament structure.

During the process of contraction, a movement of Tm of 10 to 15 Å relative to actin occurs (Haselgrove, 1972; Huxley, 1972 and Parry & Squire, 1973) suggesting that there are two separate binding sites for each actin monomer, one for the on-state and the other for the off-state. The occurrence of the 14 bands presented the idea that each of them may be actin binding sites in which alternate bands have different functions. The gross features of the alternating bands, α and β, are very similar but there appears to be subtle systematic differences in the distribution of the charged residues in these bands.

The existence of the alternating α and β bands in Tm finds support in the 3 dimensional reconstructions of actin - Tm complex in the inhibited and active states (in the presence and absence of TnT and TnI respectively) by Wakabayashi, *et al.*, (1975). In the active state (Fig. 9), the boot-shaped actin monomers on one strand of the helix are positioned such that their broad heel ends are in close contact with the Tm polymer on that side of the groove (homostrand contact) and the narrow toe ends of the actin monomers of the other strand of the helix make a more localized contact (heterostrand contact) with the same Tm

Fig. 9a. Model of the three dimensional reconstruction of actin and tropomyosin complex in the active "on" state.
[From Wakabayashi, *et al.*, 1975].

Fig. 9b. Sketch of the complex illustrating the interaction of the actin molecules with specific regions of tropomyosin.
[From McLachlan & Stewart, 1976a].


55 Å
α
β
α
β
α
β
α
β
α
β

at positions between the homostrand contacts. In the inhibited state, the homostrand interaction becomes more extensive while the heterostrand contacts come apart. McLachlan & Stewart (1976a) suggested that the α band which shows more definite structural regularities than the β band represents the homostrand binding site while the β band represents the heterostrand binding site.

Currently, the nature of the interactions between actin and Tm is not yet established. These interactions may be due to electrostatic forces between oppositely charged regions in these molecules or they may involve Mg^{++} bridges between negatively charged regions since it has been demonstrated that Mg^{++} is required for binding of actin and Tm at low salt concentration. McLachlan & Stewart (1976a) also pointed out that the 29.3 Å separation between α and β bands is close to the actin helical rise and corresponds to one quarter turn twist of the supercoil relative to actin. A quarter roll of the Tm about the helix axis would present actin a set of bands different from those to which it was previously bound. This could be the basis of a very simple mechanism for control. In the presence of Ca^{++}, Tn could force the transition by turning the TnT subunit through a quarter turn about the helix and exerting a torque.

E. TROPOMYOSIN-TROPONIN INTERACTION

The other region in αTm which exhibits anomalous distribution of charged and non-polar residues in the 19.66 periodicity is the region encompassed by residues 197-217. Although this sequence falls on the acidic zone, it contains only 2 acidic residues instead of the average eight residues. In addition, it has a significantly high concentration of uncharged polar residues and a large hydrophobic surface. These observations led McLachlan & Stewart (1976b) to propose this region as the Tn binding site. Interactions between Tm and Tn have been demonstrated experimentally in various ways. Immunoelectronmicroscopic studies of uranyl acetate stained thin filament treated with ferritin labelled antibodies showed 400 Å periodic staining along the entire filament (Ohtsuki, et al., 1967) suggesting that one Tn molecule is associated with one Tm molecule with a molecular length of about 410 Å. Upon addition of Tn to Tm, the viscosity of the Tm solution increased dramatically (Ebashi & Kodama, 1965, Ebashi & Endo, 1968) and this mixture migrated as a hypersharp peak with a sedimentation constant higher than its components in the analytical ultracentrifuge (Hartshorne & Mueller, 1967). The binding of Tn to Tm was shown to be through its TnT component (Greaser, et al., 1972 and Yamamoto & Maruyama, 1973) and independent of Ca^{++} concentration (Potter & Gergely, 1974; van Eerd & Kawasaki, 1973 and Mani, et al., 1975) and of Mg^{++} concentration (Kawasaki &

van Eerd, 1972). The dependency of these interactions on ionic strength has been demonstrated by Jackson et al., (1975). The increase of specific viscosity of Tm by the addition of equimolar amounts of Tn, in the presence of 0.3M KCl was only 15% of that in 0.06M KCl solution. They have also shown that the property of Tn to enhance the viscosity of Tm could be achieved by some CNBr fragments of TnT. Identification of these fragments have localized the Tm binding site on TnT to a region within residues 71-151. About 80% of the helical content of TnT was found in this region and a correlation between loss of helical structure and binding to immobilized Tm was observed (Pearlstone & Smillie, 1977).

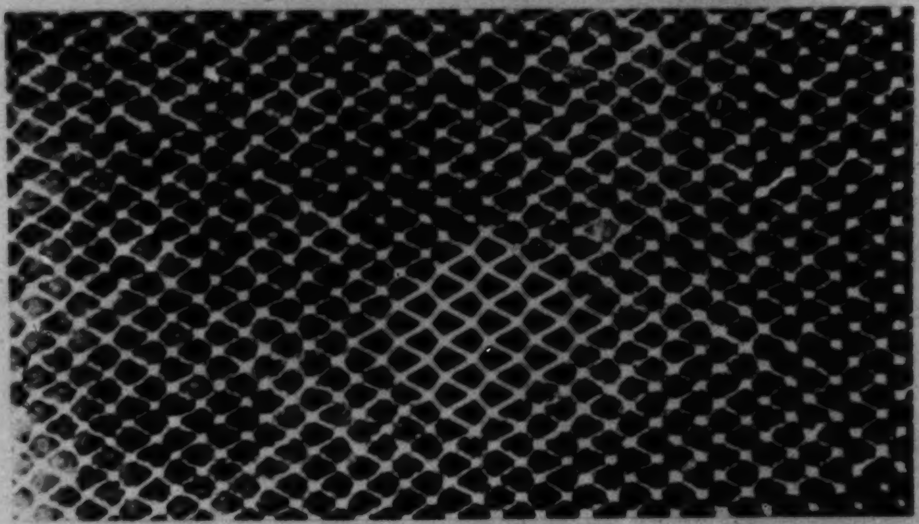
Numerous studies on ordered aggregates of Tm have been done in an attempt to delineate the Tn interaction site on Tm. Crystals with open lattice were formed by precipitation near its isoelectric pH (Bailey, 1948) while compact fibrous aggregates were obtained when precipitation was done in the presence of high concentration of divalent cations at neutral or slightly alkaline pH (Cohen & Longley, 1966).

Tm crystals are 95% water and their three dimensional structure has been determined by X-ray diffraction using data to 20 Å. The most characterized crystal (Fig. 10) has a kite-like network (Caspar, et al., 1969). It is made up of strands of head to tail polymers of Tm which cross each other forming a long arm (230 Å) and a short arm (170 Å).

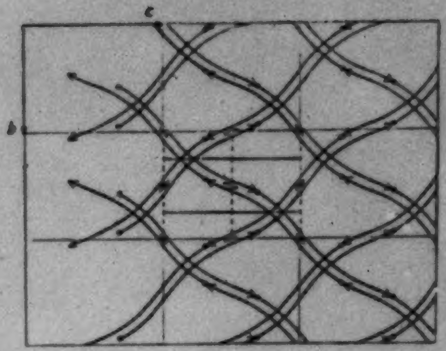
Fig. 10a. Electron micrograph of a tropomyosin crystal negatively stained with uranyl acetate. An electron density map calculated from the X-ray pattern of the hydrated crystals is superimposed on the electron micrograph.

Fig. 10b. Molecular configuration and symmetry elements in the a-axis projection of the tropomyosin crystal lattice.
[From Caspar, *et al.*, 1969].

a.



b.



The strands, which have an axial periodicity of 400 Å, are 40 Å wide indicating that they are made of two molecular filaments. The space group symmetry requires that these filaments are oppositely directed and as a consequence of their cross-over contacts, they are periodically bent. Agreement between the optical transform of the model (Fig. 10b) Caspar, et al., (1969) constructed and the X-ray diagrams of the crystals was striking.

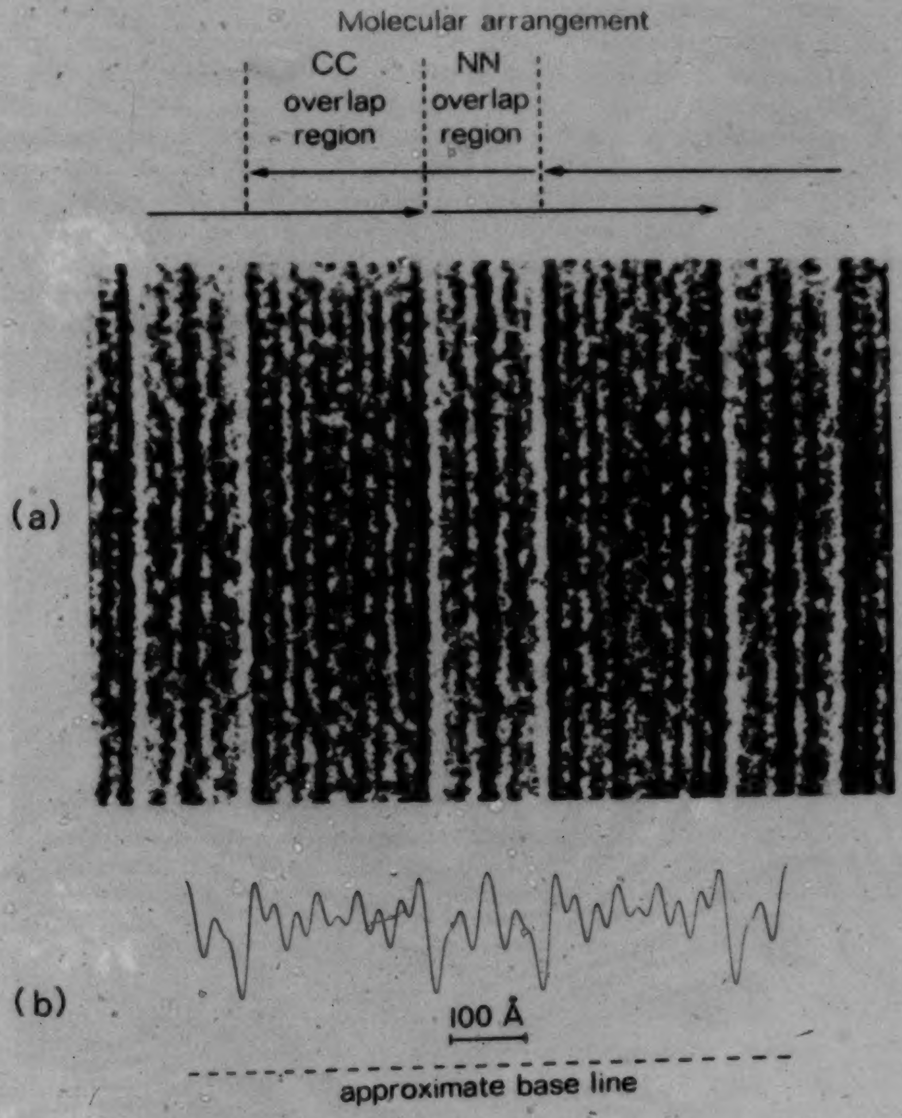
The effect of incorporation of Tn on the Tm crystals was studied by comparing X-ray diffraction patterns of crystals grown in the presence and absence of Tn. In the presence of Tn, the general arrangement of the Tm molecules were not altered but significant difference in the unit cell dimensions and in the intensities of some reflections were observed (Cohen, et al., 1972). The magnitude of the intensity changes indicated that there was only limited binding of Tn to Tm in these crystals. Both electron microscopy (Higashi & Ooi, 1968) and X-ray studies (Cohen, et al., 1972) of these crystals showed the localization of Tn in the middle of the long arm of the kite-like mesh.

Crystals of mercury derivatives of Tm allowed the placement of the entire sequence of Tn in the filament and located the mercury atom on the cysteine residue of the molecule in the middle of the long arm (Philips, et al., 1978). This suggests that the Tn binding site is near the unique cysteine residue of Tm.

Precipitation of Tm in the presence of divalent cations at neutral or slightly alkaline pH results in the formation of a variety of microscopic fibrous aggregates which are remarkable in the uniformity and regularity of their appearance (Cohen & Longley, 1966). The aggregates display clear periodicity in the longitudinal direction only, for which reason they are frequently called tactoids or paracrystals. Under the electron microscope, uranyl acetate stained paracrystals exhibit distinct banding patterns (Fig. 11) generally with a period of 400 Å. The paracrystals obtained from precipitation of Tm in the presence of high concentration of Mg^{++} are most characterized. The repeating unit in these paracrystals is a wide band of about 265 Å and a narrow band of about 135 Å separated by distinct boundaries. This pattern has dyad axes spaced about 200 Å apart positioned at the centers of the wide and narrow bands. Ohtsuki (1974) has established that one end of Tm molecule terminates at either edge of the narrow band. With the use of mercury derivative of Tm, the orientation of the Tm molecule in the paracrystals was established and the dyad axes located at 140 Å from the C-terminus and 70 Å from the N-terminus (Stewart, 1975a).

Stewart and McLachlan (1976) further studied the structure of the magnesium paracrystals of αTm by comparing their uranyl acetate stained banding pattern with the distribution of acidic residues in the amino acid sequence. It was observed that the molecular packing is such that negatively charged zones of the 14 bands with 19.66 periodicity,

Fig. 11. Magnesium paracrystals of α tropomyosin stained with uranyl acetate. The arrangement of the molecules in the paracrystal is shown above the micrograph, where arrow heads represent the c-termini of the molecules.
[From Stewart & McLachlan, 1976].


Molecular arrangement
CC overlap region
NN overlap region
(a)
(b)
100 Å
approximate base line

discussed earlier, of one Tm filament lie opposite those of neighboring antiparallel Tm filament. This arrangement suggests that a major source of stability in the paracrystals is the formation of magnesium bridges between the clusters of negatively charged zones on adjacent filaments.

Addition of Tn or TnT to the paracrystals resulted to the appearance of a light band of about 100 Å wide (in the case of Tn) at the central region of the broad band (Nonomura, et al., 1968, and Cohen, et al., 1972) suggesting that Tn or TnT is located at about one-third from one end of the molecule which was later defined to be the C-terminus (Ohtsuki, 1974 and Stewart, 1975a).

Further analysis of electron micrographs of magnesium paracrystals stained with uranyl acetate narrowed down the Tn binding region to be within 30 Å of the cysteine residue (Stewart & McLachlan, 1976).

In contrast to the conclusion that the Tn interaction site on Tm is one-third from the C-terminus of the molecule, there are reports that other regions are involved. However, these findings are not based on evidence which is so fully characterized as the investigations mentioned previously. Precipitation of Tm at low pH in the presence of TnT instead of Tn yielded crystals with open lattice of hexagonal network which were not observed with the latter (Greaser, et al., 1972, and Yamaguchi, et al., 1974). TnT was observed to bind at the intersection of the strands suggesting that it bound to the ends of the molecule. Paracrystals grown in the presence of the polyanion, dextran sulfate, exhibited a 540 Å periodicity (Ohtsuki, 1974), a pattern arising from head to head aggregation of Tm molecule. The polyanion, which complexes with the cationic regions of Tm, bridges two molecules through their N-termini. These paracrystals did not bind to Tn or TnT and in conjunction with results obtained from structural analysis of Tm paracrystals, Hurtwiz and Walton (1977) interpreted this to mean that Tn binds to basic residues and postulated the acid poor regions either residues 86-96 or 179-186 to be the Tn binding site. Recently, Ueno and Ooi (1977) prepared fragments of αTm, N(1-189), C(190-284), S(13-128 or 149) and P(183-284) by specific chemical cleavage and tryptic digestion. Individually, none of these fragments bound to Tn but mixtures of NH_2-terminal and COOH-terminal fragments exhibited binding. On this basis, they postulated the binding site to be at residues 149-190.

F. AIMS OF THIS PROJECT

Although models of the mechanism and regulation of contraction have been proposed, a complete understanding of the phenomenon will only be achieved upon characterization of the proteins involved and their interactions at the molecular level. To this end, the primary structures of all the thin filament proteins have been determined and their properties studied. However, more information about the nature and positions of the binding sites of these proteins and the correlation of their structure and function is still required. This study was undertaken with the hope of providing more information about the structure of Tm, which is the structural link between Tn and actin, and of delineating its interactions with the other thin filament proteins.

Circular dichroism studies on Tm in the absence of denaturants below 25°C suggested that it is almost completely α helical (Cohen & Szent-Gyorgyi, 1957, and Wu & Yang, 1976). However, the presence of regions of less ordered structure were indicated by denaturation studies (Woods, 1969, and Pont & Woods, 1971) and the formation of trypsin resistant fragments upon prolonged exposure of Tm to the enzyme (Eckard, 1973). The localization of these regions in the molecule were determined, in the present study, by investigating the modes of action of the proteolytic enzymes, trypsin and chymotrypsin, on Tm and by analyses of the primary structure of the molecule.

While this study was in progress, information about the localization of these less helical regions were reported in the literature. Parry (1975) did a conformational analysis on the sequence of αTm, using the conformational parameters listed by Chou & Fasman (1974). The parameters of the amino acids were smoothed with a Gaussian function with a half width of 4 residues and plotted against their positions in the sequence. The results indicated that the

the nature and positions of the binding sites of these proteins and the correlation of their structure and function is still required.

Although Tm has been generally viewed as being completely and uniformly helical (Cohen & Szent-Gyorgi, 1957; Wu & Yang, 1976), denaturation studies have indicated the occurrence of regions of varying stability (Woods, 1969; Pont & Woods, 1971). This study undertaken with the hope of defining these regions by investigating the action of the enzymes, trypsin and chymotrypsin, on Tm during limited proteolytic digestion and by determining the helical contents and stability to heat denaturation of large fragments of Tm.

Another objective of this study was to delineate the interactions of Tm with the other thin filament proteins, Tn and F-actin. Large fragments of αTm, which still retain the basic properties of the molecule were prepared and their binding properties to Tn and its components and F-actin were studied by various methods.

CHAPTER II

EXPERIMENTAL METHODS

A. COLUMN CHROMATOGRAPHY

1. Gel Filtration Chromatography

Sephadex G-75, swelled in 10% formic acid, was packed in two Pharmacia columns (5 x 100 and 5 x 150 cm) connected in series in such a way that the short column was subjected to downward flow and the long column to upward flow. The tryptic digest of αTm was directly applied to the short column and eluted at 60 ml/hr, maintained by a Beckman Accu Flo constant volume pump at room temperature. Fractions of 20 ml were collected.

Protein solutions containing urea were desalted in a Sephadex G-25 column (2.5 x 100 cm) equilibrated and eluted with 5% formic acid at 30 ml/hr at room temperature.

In both cases, fractionation was monitored by alkaline hydrolysis of aliquots of the fractions collected and the quantity of amino acids in the hydrolysates were determined from their absorbance at 570 nm after reaction with ninhydrin (Hirs, et al., 1956 and Moore, 1954).

2. Ion Exchange Chromatography

The cation exchanger, CM-32 cellulose (Whatman), was packed in 1.2 x 50 cm column and equilibrated with 8M urea, 50 mM sodium formate, 0.25 mM DTT, pH 4.0 buffer at room temperature. The buffer was pumped through the column at 6 ml/hr for about an hour after the application of the sample before a linear gradient of 600 ml 0 - 0.2M NaCl was established.

In some experiments an anion exchanger, Sephadex QAE A50, was used. The column (2 x 25 cm) of resin was equilibrated with 0.1M KCl, 8M urea, 50 mM Tris, pH 7.5 solution at 13 ml/hr at room temperature. The polypeptide fragments were eluted with a 500 ml gradient of 0.1 - 0.4M KCl.

The elution profiles, in both cases, were monitored by measuring the absorbance at 280 nm of the 10 ml fractions.

B. CHEMICAL METHODS

1. Amino Acid Analyses

Amino acid analyses were performed on a Durrum model D500 or Beckman model 120C. A fired test tube, containing the freeze dried material dissolved in constant boiling HCl with 0.1% phenol ~~was~~ evacuated, sealed and incubated at 110°C for 22 hours. Quantitative determination of threonine, serine, valine and isoleucine required incubation of some samples at 48, 72 and 96 hours. The hydrolysate was dried over NaOH pellets in a vacuum desiccator.

Cysteine is degraded during acid hydrolysis. Its determination necessitated its conversion to cysteic acid with performic acid as described by Moore (1963).

The exact concentration of a protein with known amino acid composition can be calculated from the determination of stable amino acids such as leucine and alanine in a known volume of the protein solution which has been freeze dried and hydrolyzed.

2. NH_2-terminal Sequence Determination

Automatic Edman degradations of the polypeptides were made with a Beckman 890B sequencer using the standard single coupling, double cleavage procedure (Edman & Begg, 1967). The thiazoline derivatives produced were converted into phenylthiohydantoins by reaction with 0.2 ml 1M HCl at 80°C for 10 minutes (Edman & Begg, 1967). The phenylhydantoins were identified by gas liquid chromatography in a Beckman GC 45 gas chromatograph (Pisano & Bronzert, 1969) and by analysis on a Durrum D500 amino acid analyzer following hydriodic acid hydrolysis (Smithies, et al., 1971).

3. COOH-terminal Sequence Determination

Penicillocarboxypeptidase S1 (gift from Dr. T. Hofmann) was used to sequence the COOH-termini of some fragments of αTm. The digestion was carried out according to the procedure of Hui, et al., (1974) with some modifications. About 80 nmoles of peptides were dissolved in 160 µl of 0.2M pyridine, 0.25M HCOOH, pH 4.2 solution and digested with the enzyme at 37°C at an enzyme to substrate ratio of 1:1000. Samples (25 µl) were taken at different time intervals and mixed with 25 µl of 0.4 mM norleucine, 0.43M HCl solution.

The reaction was quenched by heating the mixture at 100°C for 5 minutes. Quantitative determinations of the amino acids released with time were done on the Durrum D500 amino acid analyzer.

The peptides insoluble under the above conditions were sequenced using carboxypeptidase-A-DFP (Sigma). A sample (25 µl) of the carboxypeptidase-A-DFP suspension was solubilized in a solution of 25 µl of 1% $NaHCO_3$ and 35 µl of 0.1M NaOH (Johnson & Perry, 1968). The enzyme solution (20 µl) was immediately pipetted into a solution of 1 mg peptide in 200 µl of 50 mM sodium phosphate buffer, pH 7.8 at room temperature. Aliquots (25 µl) taken at various times were added to 25 µl of 0.4 mM norleucine, 0.96M HCl solution, which immediately lowered the pH to about 2.2 and terminated the reaction. The free amino acid contents of the aliquots were determined directly on the Durrum D500 amino acid analyzer.

C. PHYSICAL METHODS

1. SDS-polyacrylamide Gel Electrophoresis

Slab polyacrylamide gel (8-10% w/v) electrophoresis (Akroyd, 1967) was performed in the presence of 0.1% SDS in 100 mM sodium phosphate buffer pH 7.0 as described by Weber and Osborn (1969). To differentiate Tm and some of its fragments from proteins of similar molecular weights, 6M urea was incorporated in the gel (Sender, 1971). Protein samples (1 mg/ml) for both SDS and SDS-urea gels were prepared by dissolving the protein in 1% SDS, 6M urea, 1% mercaptoethanol, 50 mM sodium phosphate, pH 7.0 buffer and incubated at 60° for 30 minutes. Bromophenol blue marker was added to the samples before application to the gel. At the end of the run, the gel was washed with 10% methanol, 10% acetic acid solution for at least 1 hour before staining with 0.25% Coomassie blue R250 in 10% acetic acid, 50% methanol solution for half an hour. Destaining of the gel was accomplished in 10% methanol, 10% acetic acid solution at room temperature.

2. Circular Dichroism Measurements

(a) α-Helical Content Determination

A Cary model 6001 CD attachment to a Cary 60 recording spectropolarimeter was used for circular dichroism measurements according to the procedure described by Oikawa *et al.*, (1968). The mean residue ellipticity, $[\theta]_\lambda$, at a particular wavelength, λ, was calculated from the following equation (Freifelder, 1976).

$$[\theta]_\lambda = \frac{\theta obs\ m}{100\ \ell c}$$

where

m is the mean residue weight (taken to be 115 in this study)

θobs is the ellipticity value at the wavelength, λ, of interest

ℓ is the light path length in cm

c is the protein concentration in g/cm^3.

The fractional helix, f_H, values at 222 nm at 10°C and 27°C were calculated from the following relationship (Cassim & Yang, 1967), using the parameters of Chen et al., (1974).

$$f_H = \frac{[\theta]_{222} - [\theta]_{r.c.}}{[\theta]_H - [\theta]_{r.c.}}$$

where

$[\theta]_{222}$ is the mean residue ellipticity at 222 nm of the peptide

$[\theta]_{r.c.}$ is the mean residue ellipticity of a 100% random coil peptide at 222 nm (taken as +1580 in this study)

$[\theta]_H$ is the mean residue ellipticity of a 100% α helical peptide (taken as -39500 in this study).

(b) Melting Temperature Determination

The ellipticity melt experiments were performed by increasing the temperature of the protein solution stepwise from 10°C to 70°C in a water jacketed thermoregulator. The ellipticity at 221 nm was measured 15 minutes after the temperature has been raised to insure that the sample had attained the desired temperature. The temperature intervals were not equally spaced throughout the run but rather close in the transition region. The reversibility of the process of denaturation was determined by measuring the ellipticity of the sample as the temperature was lowered stepwise.

The protein samples for the CD experiments were prepared by extensive dialysis of about 1 mg/ml polypeptide solution against 0.1M KCl, 1 mM DTT, 50 mM sodium phosphate, pH 7.0 buffer. The concentrations of the solutions were determined by amino acid analysis.

D. INTERACTIONS OF αTm AND ITS FRAGMENTS WITH Tn AND F-ACTIN

1. Interactions with Tn and its Components

(a) Ultracentrifugation

Sedimentation velocity experiments were carried out at 20°C in a Beckman model E analytical ultracentrifuge. The sample was mounted in a cell assembly with a Kel-F, 2° -12 mm centerpiece and plain quartz windows. The operating speed was 60,000 rev/min and pictures were taken at a bar angle of 65° with a Schlieren optical system.

(b) Polyacrylamide Gel Electrophoresis

TnT and TnC used in the experiments were prepared from the fractionation (Greaser & Gergely, 1971) of the troponin complex extracted by the combined methods of Ebashi, *et al*., (1971) and Staprans, *et al*., (1972). TnT was dissolved in 0.1 mM HCl and dialyzed against 0.5M KCl, 10 mM imidazole, 1 mM DTT, 0.01 mM $CaCl_2$, pH 7.0. TnC was dissolved and dialyzed against the same buffer for 24 hours. The protein concentrations of the dialyzed solutions were determined spectrophotometrically employing the values $E^{1\%}_{1cm,\ 280\ nm}$ = 5.0 for TnT (Wilkinson, 1974) and $E^{1\%}_{1cm,\ 277.5}$ = 2.3 for TnC (Murray & Kay, 1972). TnCT complex was prepared by mixing TnC and TnT in a 1:3 molar ratio. The resulting solution was dialyzed against 0.15 M KCl, 1 mM DTT, 0.01 mM $CaCl_2$, 10 mM imidazole pH 7.0 for 24 hours at 4°C.

αTm or its fragments and TnCT or CB1, the αTm binding fragment of TnT (Jackson, et al., 1975 and Pearlstone & Smillie, 1977), were combined in molar ratios of 1:0.75, 1:1.5 and 1:3. Since the ratio of the components of TnCT was 1 mole TnC to 3 moles TnT, the calculation for mixtures with αTm was based on the amount of TnC in TnCT. The mixtures were incubated for about 12 hours at 4° before assaying for binding.

Slab polyacrylamide gel electrophoresis in benign medium, adapted from the procedure of Stone and Perry (1973), was used to detect the formation of a complex between the components of the mixture. The gel contained 6% (w/v) cyanogum 41, 40% (v/v) glycerol, 20 mM Tris, 125 mM glycine pH 8.6 buffer. The sample applied to the gel was prepared by the addition of glycerol to 40% to the binding mixture. The electrode buffer was 0.1M KCl, 1 mM $CaCl_2$, 20 mM Tris, 125 mM glycine pH 8.6. Each run was carried out at 60 mA for 11 hours.

Identification of the components of the new complex formed was done by cutting out the band from an unstained portion of the preparative gel and electrophoresing it in a SDS-urea polyacrylamide gel or urea polyacrylamide gel. The urea gel was prepared and run in the same manner as the benign gel except for the omission of glycerol and the incorporation of 6M urea both in the gel and electrode buffer.

The cut out band was soaked in the electrode buffer in a dialysis bag (Spectrapor membrane tubing, molecular weight cut off = 3500) and dialyzed against the buffer at 34°C for 36 hours. The cut out band was rinsed and placed on top of the second gel and electrophoresed. The solution in which the gel was soaked was also applied to the second gel to check for diffusion of proteins from the cut out band.

(c) Gel Filtration Chromatography

Biogel A 0.5 m (Biorad) packed in 0.9 x 105 cm column was equilibrated with the eluent, 0.15M KCl, 1 mM DTT, 0.01 mM $CaCl_2$, 10 mM sodium phosphate buffer, pH 7.0 at 3.5 ml/hr. The sample (2 mg) in 1 ml of the buffer was applied to the column. αTm, its fragments and CB1 were run through the column individually as well as mixtures of αTm or its fragments and CB1 at molar ratios ranging from 0.5:1 to 2.5:1 (αTm:CB1). The mixtures were incubated at 4°C overnight before application to the column. Elution was monitored by taking the absorbance of the 1 ml fractions at 230 nm. The composition of the eluted peaks was analyzed in a SDS-urea gel.

(d) Affinity Chromatography

Tn and CB1 were coupled to aminohexyl (AH) and carboxyhexyl (CH) Sepharose 4B (Pharmacia) according to the procedure recommended by the supplier. The resin (1g), swelled in 400 ml of 0.5 M NaCl solution at room temperature overnight, was filtered in a glass sintered funnel and washed with 200 ml of 0.5M NaCl and 400 ml of distilled water. Tn or CB1 (10 mg), dissolved in 5.5 ml distilled water, was added to the washed resin after its absorbance at 230 and 280 nm was determined. A solution of EDC, 1-ethyl-3-(3-Dimethylaminopropyl)-carbodiimide hydrochloride, (133 mg in 0.5 ml water) was added dropwise to a final concentration of 1M to the slurry, the pH of which was adjusted to 4.5. The pH of the mixture was maintained at 4.5 with HCl for 1 hour. The reaction was then allowed to proceed at room temperature for 29 hours with gentle continuous end over end mixing. At the end of the reaction time, the resin was filtered and washed with 10 ml of 1M NaCl, 50 mM sodium acetate pH 4.0 buffer. The absorbance at 230 and 280 nm of the combined filtrates were measured. The resin was washed with 100 ml more of the pH 4.0 buffer and then with 10 ml of 1M NaCl, 50 mM NH_4HCO_3 pH 8.5 and 100 ml of the same buffer. The absorbance of the 10 ml filtrates at 230 and 280 nm was recorded. The resin was further washed with two alternating cycles of 100 ml each of the acidic and basic buffers and finally with 400 ml of water. The resin, equilibrated with 10 mM imidazole, 1 mM DTT, 1 mM EGTA, pH 7.0, was packed in a 0.9 x 8 cm column.

The preparation of affinity columns of Tn, TnT and CB1 with CNBr activated Sepharose 4B (Pharmacia) was performed as suggested by the supplier. The resin (1.5 g), swelled in 1 mM HCl, was coupled to the desired protein

(15 mg) in 7.5 ml of 0.1M $NaHCO_3$, 0.5M NaCl solution. The coupling reaction was allowed to proceed at room temperature for 2 hours with gentle continuous end over end mixing. The reaction was terminated by filtering the resin and washing it with 10 ml of the coupling buffer. The absorbances of the filtrates at 230 and 280 nm were determined and washing of the resin was continued with 200 ml of the buffer. Deactivation of the unreacted groups in the resin was accomplished by gentle mixing of the resin with 7.5 ml of 1M ethanolamine pH 8.0 for 1.5 hours. Non-covalently bound proteins were removed by washing the resin with 1M NaCl, 0.1M sodium acetate pH 4.0 and 1M NaCl, sodium borate pH 8.0 buffers, alternately, in the same fashion as in the preparation of the CH and AH sepharose-Tn resins.

Rough quantitative estimates of the coupled proteins were done from the differences of the absorbances at 230 and 280 nm of the protein solutions and the filtrates obtained from washing the resin.

The column was washed with at least 10 ml of the eluent, 1 mM DTT, 1 mM EGTA, 10 mM imidazole, pH 7.0, before the sample (about 0.5 mg of peptide in 0.25 ml buffer) was applied to the column. αTm and its fragments were applied to the column individually as well as mixtures of equal weights (about 0.5 mg) of two fragments. The eluent was then passed through the column at 3.5 ml/hr for about 3 hours before a 60 ml linear gradient of 0-0.5M KCl was established. All the experiments were done at 4°C. The elution profile was obtained from the absorbances of 1 ml fractions at 230 nm and the salt concentrations of selected fractions determined with a Radiometer type CDM 2e conductivity meter. The composition of the eluted protein peaks was determined by SDS urea gel electrophoresis of the dialyzed and freeze dried pooled fractions.

2. Interaction with F-actin

(a) Cosedimentation Method

The assay for binding of αTm or its fragments to F-actin was patterned after the procedure of Eaton, et al., (1975). αTm or its fragment (0.4 mg) was dissolved in 1.34 ml of 30 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 2 mM ATP, 1 mM imidazole pH 7.0 and centrifuged at 20,000 g for 7 hours at 4°C to remove undissolved particles. The supernatant was mixed with 2 mg F-actin (3 mg/ml) prepared by the procedure of Spudich & Watt (1971) and incubated at room temperature for 2 hours. The mixture was centrifuged at 20,000 g for 15-20 hours at 4°C. The supernatant was dialyzed against water overnight and freeze dried. The pellet was carefully rinsed with water to remove contaminating supernatant solution and then dissolved in H_2O and freeze dried. The compositions of the pellet and supernatant were determined by SDS polyacrylamide gel electrophoresis.

The validity of the assay was tested by determination of the amount of αTm that bound to actin by scanning the gel of the pellet obtained from the reaction. The amounts of actin and αTm were calculated from their corresponding standard curves, constructed by plotting the intensities of the Coomassie stained protein band against the amount of protein applied to the gel. This was accomplished by dissolving αTm and F-actin in 0.5% HCOOH and dialysing against the same solution. Aliquots of the solutions were taken for concentration determination by amino acid analysis and 1 ml aliquots were freeze dried. The freeze dried samples were dissolved in known volumes of 6M urea, 5% SDS, 1% mercaptoethanol, 50 mM sodium phosphate pH 7.0 and heated at 60°C for half an hour. Various volumes of the samples were applied to 5 mm disc gels. The destained gels were scanned at 590 nm.

CHAPTER III

PREPARATION AND ISOLATION OF αTm AND ITS FRAGMENTS

Rabbit skeletal Tm was initially prepared by K. Bailey (1948). The protocol for the extraction and purification was based on isoelectric precipitation of Tm followed by $(NH_4)_2SO_4$ fractionation. The method has been studied in great detail and improved by Hartshorne and Mueller (1969). In this study, slight modification in the procedure was further introduced to obtain a more homogeneous protein.

Tm is intimately involved with the other thin filament proteins, Tn and actin. Various large fragments of αTm were prepared for experiments designed to further our understanding of the interactions between these proteins. The methods for the preparation of the fragments of αTm were chosen on the basis of the specificity of the sites of cleavage of peptides by the enzymes trypsin, chymotrypsin and carboxypeptidase A as well as the chemical reactions of CNBr and the combined action of DTNB and KCN.

A. α TROPOMYOSIN

Muscle powder was prepared from either frozen or freshly minced rabbit skeletal muscle by the procedure of Bailey (1948) except that the muscle fiber was washed with acetone instead of ether as the final step. The air dried muscle powder was stored at -20°C.

Tm was isolated from the acetone muscle fiber using a modified procedure of Bailey (1948). Acetone powder (100g) was dispersed in 700 ml of 1.0M KCl, 0.5 mM DTT and adjusted to pH 7.0 with 1M NaOH. The mixture was allowed to stand, with occasional stirring, at pH 7.0, 25°C for 16 hours. The liquid was then removed from the residue by passage through two layers of cheese cloth and the residue re-extracted with 1.0M KCl, 0.5 mM DTT for 2 hours. The subsequent procedure was done at 0°C. The pH of the combined extracts was adjusted to pH 4.6 with 1M HCl and the solution was stirred for half an hour. The precipitate was spun down, dispersed in 1ℓ of the extraction solution and neutralized to pH 7.0. Undissolved particles were removed by centrifugation. Precipitation at pH 4.6 was repeated. The pellet was dissolved in 1ℓ of the extraction buffer at pH 7.0 and the pH readjusted to 4.3 with 1M HCl. The precipitate was dissolved in 0.5 mM DTT solution and neutralized to pH 7.0 with 1M NaOH. Solid $(NH_4)_2SO_4$ was added slowly, with stirring, to the solution to a concentration of 54% saturation. Maintaining the pH at 7.0, the solution was stirred for half an hour and the precipitate was spun down. More solid $(NH_4)_2SO_4$ was added to the supernatant to a final concentration of 65% saturation. The solution was centrifuged after stirring for 30 minutes at pH 7.0. The precipitate was dissolved in 200 ml of 0.5 mM DTT and dialyzed extensively against 10 mM mercaptoethanol at 4°C before it was lyophilized.

Tm was fractionated into its α and β components on a

CM-32 cellulose column at pH 4.0 in the presence of 8M urea (Cummins & Perry, 1973).

Rabbit cardiac Tm is a homogeneous protein as judged from SDS gel electrophoresis. Its electrophoretic mobility and primary sequence are identical to rabbit skeletal αTm (Lewis & Smillie, unpublished). Cardiac Tm was used to substitute for rabbit skeletal αTm in the interaction experiments. A major advantage using cardiac Tm is that it was not exposed to denaturing conditions in its preparation. Cardiac and skeletal Tm were prepared in the same manner but the latter required fractionation in the presence of 8M urea to isolate pure αTm.

B. FRAGMENTS OF αTm

1. Digestion with Trypsin [3.4.4.4]

When this work was initiated, Eckard (1973) had previously reported the preparation of two trypsin resistant core peptides by exposure of Tm to trypsin at an enzyme ratio of 1:100 at pH 8.5, 0°C for 24 hours. Based on the histidine contents and the results from carboxypeptidase-A digestion of the peptides, it was suggested that one of the peptides was derived from the middle part of the Tm molecule while the other came from the COOH-end of the molecule.

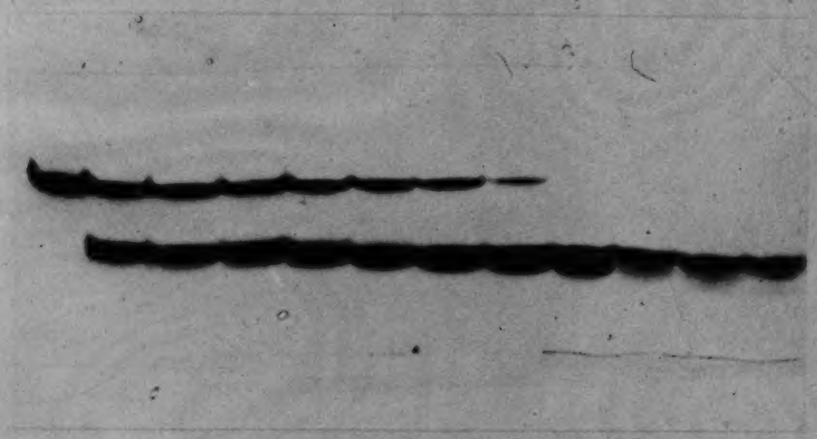
To gain a better understanding of the action of trypsin on Tm, the activity of the enzyme was investigated in greater detail. The time course of the digestion at 0°C, pH 8.5 was studied at the following enzyme to substrate ratios; 1:1000, 1:500, 1:200, 1:100, 1:50, 1:33. A stock solution of trypsin TPCK (Worthington Biochem. Corp.) was prepared by dissolving the enzyme in 1 mM HCl and dialyzing it against the same solution to remove contaminating salts and/or metal ions. The substrate was an 8 mg/ml solution of αTm in 50 mM NH_4HCO_3, pH 8.5 dialyzed against the same buffer overnight. The concentrations of the enzyme and αTm solutions were determined spectrophotometrically using the values $E^{1\%}_{280\ nm,\ 1\ cm} = 14.4$ for trypsin (Worthington Manual, 1972) and $E^{1\%}_{277\ nm,\ 1\ cm} = 3.3$ for αTm (Woods, 1967). The enzymic digestions were carried out at 0°C, pH 8.5 and the products of digestion at various times were examined on SDS polyacrylamide gels both in the presence and absence of 6M urea. For a reason which is not clear, Tm and some of its fragments do not exhibit electrophoretic mobilities proportional to their molecular weights in SDS gels in the presence of urea. This anomalous property has been exploited to differentiate them from other proteins or fragments which have the same or similar molecular weights. The samples for the gels were prepared as follows: 0.1 ml aliquots of the digest were taken at different time intervals and added to 0.3 ml of 9.6M urea, 60 mM HCl pH 2.9. The combined action of low pH and urea quenched the reaction immediately. In previous experiments, it had been observed that lima bean inhibitor was not effective in terminating the reaction instantly. The pH of the solution was raised by addition of 133 μl of 0.2M sodium phosphate buffer pH 7.0 containing

4% SDS and 5 µl of 2M NaOH. The resulting solutions were heated at 60°C for 30 minutes before application of 20 µl to the gels. The products of tryptic digestion at increasing reaction time at an enzyme to substrate ratio of 1:200 are demonstrated on the SDS and SDS-urea gels shown in Fig. 12a and b. The SDS gel showed that as the tryptic digestion time was increased, the disappearance of the intact αTm was accompanied by increasing amounts of only two lower molecular weight bands. However, the SDS-urea gel showed a different pattern. At very short digestion time, two bands were observed but as time progressed, new bands appeared and others disappeared.

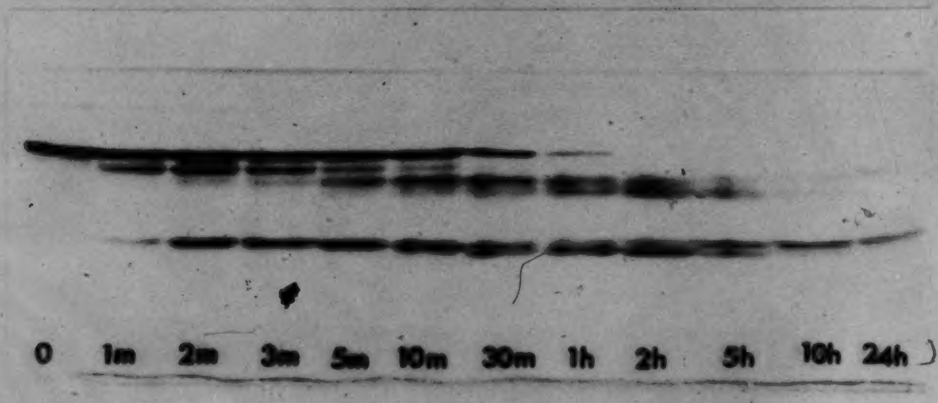
To determine whether the two early bands were due to a single point cleavage of Tm by the enzyme, aliquots of the digest were taken and added to 10% TCA solution to precipitate Tm and the large peptides. SDS and SDS-urea gels demonstrated that undigested Tm as well as the fragments observed in the whole digest at short digestion time were completely precipitated by the acid. The supernatants, which should have contained small peptides and/or free amino acids, were washed with an equal volume of ether five times to extract dissolved TCA and then the clear aqueous layers were lyophilized. The composition of the freeze dried samples was determined by high voltage paper electrophoresis and by amino acid analysis of their acid hydrolysates. These studies indicated the absence of small peptides or free amino acids during the early time of digestion suggesting

Fig. 12. Time course study of tryptic digestion of αTm. SDS polyacrylamide gels in the absence (a) and presence (b) of 6M urea of the products of tryptic digestion of αTm at 0°C, pH 8.5, enzyme to substrate ratio of 1:200 at different digestion times.

a,



b.


0
1m
2m
3m
5m
10m
30m
1h
2h
5h
10h
24h

that the two fragments were the result of the hydrolysis of a single peptide bond. Study of several trials of time course digestion with various αTm preparations indicated that the best conditions for the preparation of the two large fragments were 0°C, pH 8.5 and 1 minute at an enzyme to substrate ratio of 1:200.

(a) Short Term Digestion

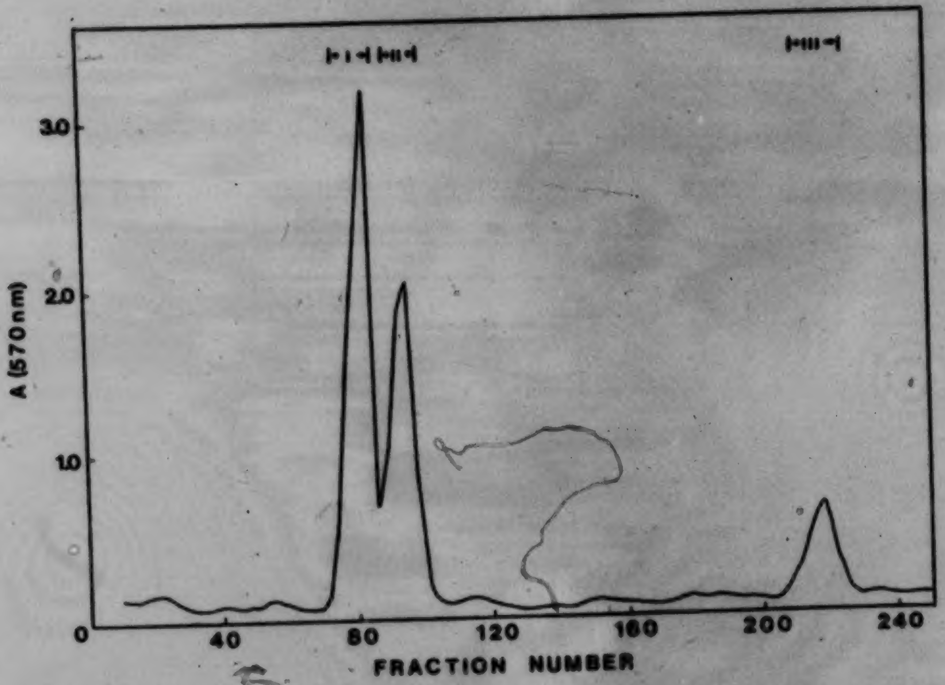
Efforts were made to isolate the large fragments produced at short digestion times. Trypsin-TPCK was added to an 8 mg/ml dialyzed solution of αTm (404.6 mg) in 0.05M NH_4HCO_3, pH 8.5 at 0°C to give a ratio of 1:200. After 1 minute, the reaction was terminated by lowering the pH with formic acid and the digest was applied directly to a column of Sephadex G-75 equilibrated with 10% formic acid. The elution profile of the fractionation (Fig. 13a) was monitored by alkaline hydrolysis followed by ninhydrin reaction of the eluate. Compositions of the pooled fractions were determined by SDS polyacrylamide gel electrophoresis (Fig. 13b). Peak I consisted of undigested αTm while peak II (119 mg) was a mixture of the two fragments.

Chromatography of 110 mg of peak II material on a cation exchanger, CM-32 cellulose, purified the peptides as shown by the elution profile (Fig. 14a) and the SDS gels (Fig. 14b). These fragments were labelled T1 and T2. The preparation and purification procedures produced 31.1 mg T1 and 40.8 mg T2, which represented 16.7 and 18.8% yields

Fig. 13a. Fractionation of products of short term digestion of αTm on Sephadex G-75. Column dimensions; 5 x 250 cm; eluent: 10% formic acid; flow rate: 60 ml/hr operated at room temperature; fraction size: 20 ml. Effluent was monitored by alkaline hydrolysis followed by ninhydrin reaction. Fractions were pooled as indicated.

Fig. 13b. SDS and SDS-urea polyacrylamide gels of fractions I and II.

a.


I
II
III
3.0
2.0
1.0
A (570nm)
0
40
80
120
160
200
240
FRACTION NUMBER

b.

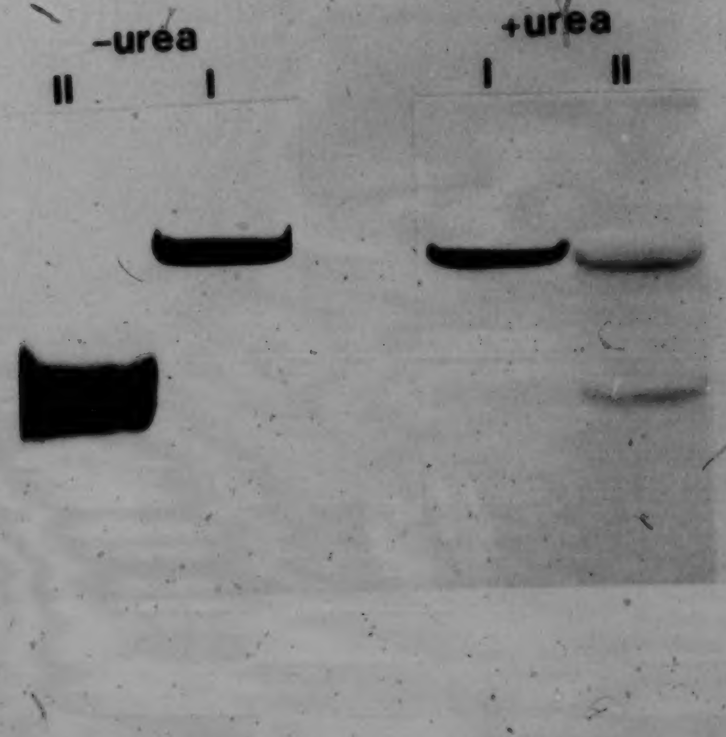
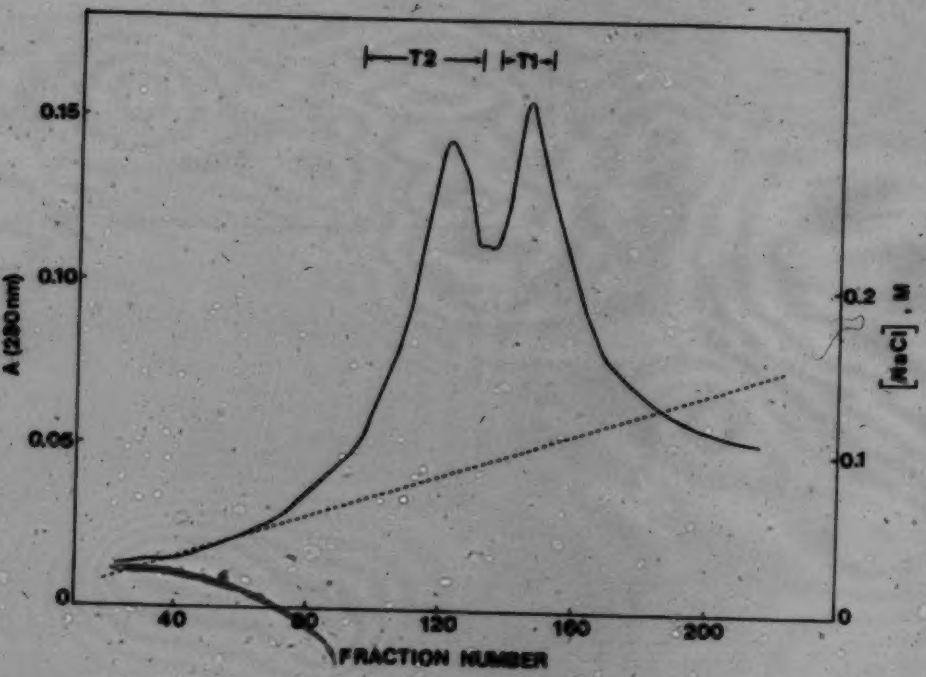

-urea
II
I
+urea
I
II

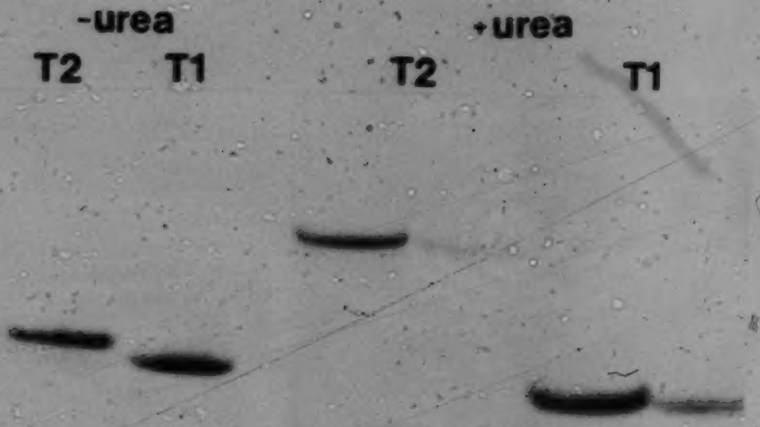
Fig. 14a. Chromatography of T1 and T2 on CM-32 cellulose. Column dimensions: 2.5 x 50 cm. Starting buffer was 8M urea, 50 mM sodium formate, 1 mM DTT, pH 4.0. Linear gradient was established with 1.5 ℓ starting buffer and 1.5 ℓ starting buffer made 0.2M with NaCl. Flow rate: 30 ml/hr operated at room temperature; fraction size: 10 ml. Effluent was monitored by absorbance at 280 nm. Fractions were pooled as indicated.

Fig. 14b. SDS and SDS-urea polyacrylamide gels of T1 and T2.

a.


T2
T1
0.15
0.10
0.05
0
A (280nm)
40
80
120
160
200
FRACTION NUMBER
0.2
0.1
0
[NaCl] , M

b.


-urea
T2
T1
+urea
T2
T1

respectively of the starting material.

(b) Long Term Digestion

Attempts were also made to isolate the trypsin resistant core peptides using the conditions of Eckard and Cowgill (1976) with slight modification. The enzymic reaction was carried out on 8 mg/ml solution of αTm (412 mg) in 0.4 M NaCl, 0.1M Tris, pH 8.5, 0°C at an enzyme to substrate ratio of 1:50 for 24 hours. The reaction was quenched with the addition of lima bean inhibitor.

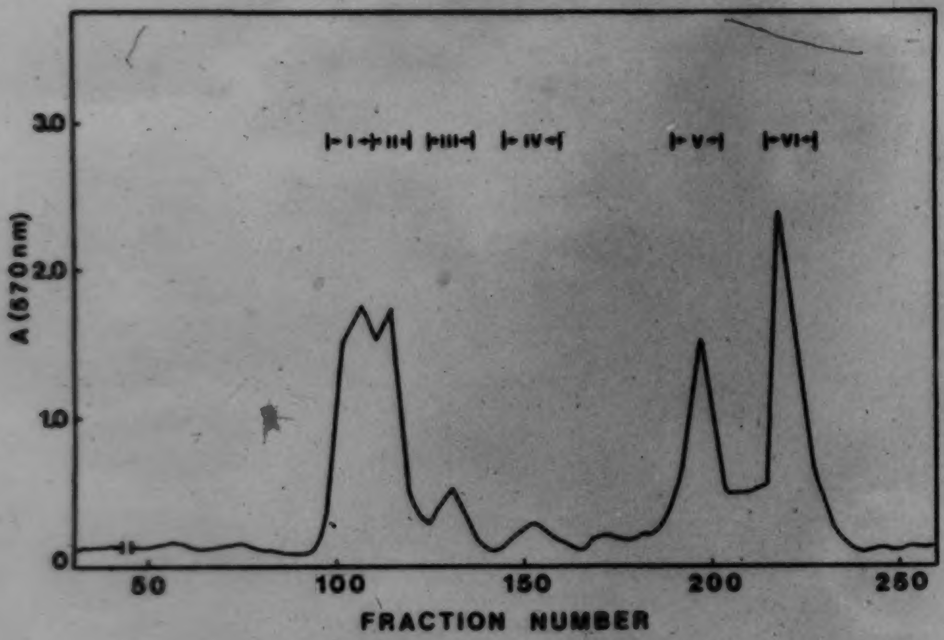
The products of hydrolysis were separated by gel filtration on Sephadex G-75 equilibrated with 10% formic acid. The elution profile (Fig. 15a) showed numerous peaks. Peak I (74.6 mg) and II (47.5 mg) were composed of 2 fragments each, as judged from SDS gels (Fig. 15b), whereas peaks III (19.9 mg) and IV (14.4 mg) showed single bands. Amino acid analysis of peak IV revealed high contents of proline and cysteine. Since αTm does not contain proline and has only one cysteine, this fraction was probably derived from the lima bean inhibitor or trypsin. When run on the SDS polyacrylamide gels, peaks V and VI gave no detectable bands and were probably composed of small peptides, free amino acids and/or Tris salt. The material from peak III (22.2% of the starting material) was labelled as T5.

The peptides of peak I were separated by chromatography on a Sephadex QAE A50 column (2.5 x 100 cm) at pH 7.5 in the presence of 8M urea (Fig. 16a and b). The yields of the

Fig. 15a. Separation of products of long term tryptic digestion of αTm on Sephadex G-75. Column dimensions: 5 x 250 cm; eluent: 10% formic acid; flow rate: 60 ml/hr operated at room temperature; fraction size: 20 ml. Effluent was monitored by alkaline hydrolysis followed by ninhydrin reaction of the fractions. Fractions were pooled as indicated.

Fig. 15b. SDS and SDS-urea polyacrylamide gels of fractions I, II, III and IV.

a.


I
II
III
IV
V
VI
3.0
2.0
1.0
0
A (570nm)
50
100
150
200
250
FRACTION NUMBER

b.

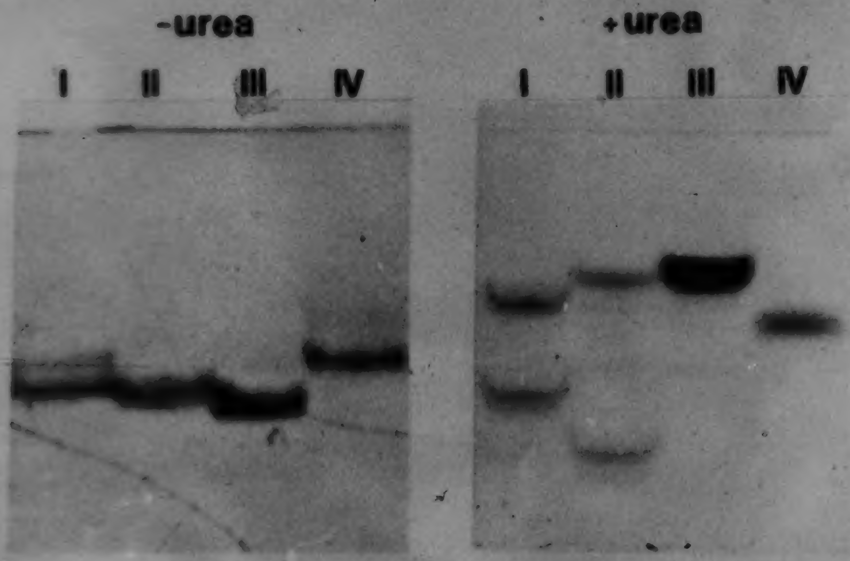
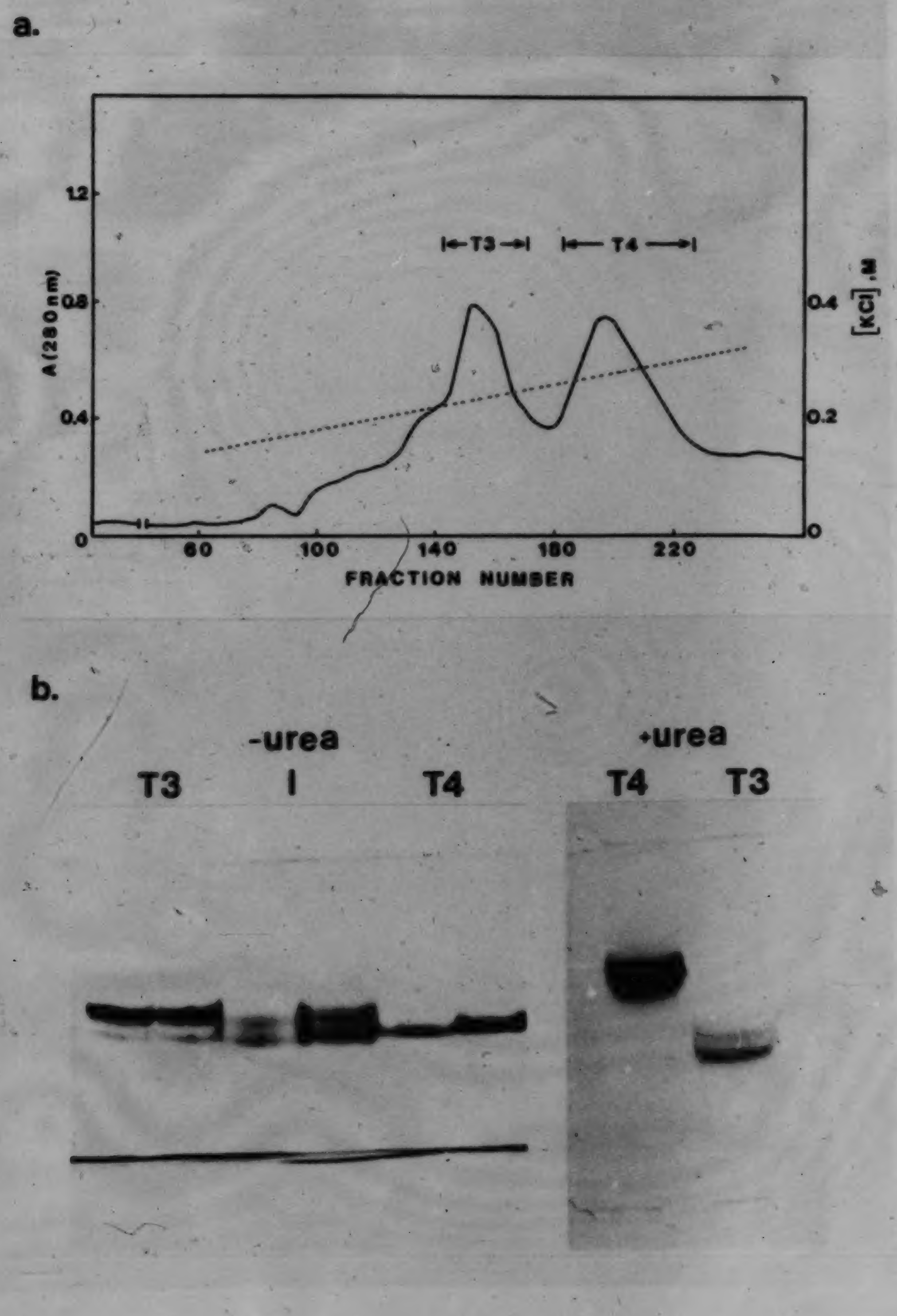

-urea
+urea
I
II
III
IV
I
II
III
IV

Fig. 16a. Chromatography of fraction I on Sephadex QAE-A50. Column dimensions: 2.5 x 100 cm. Starting buffer was 0.1M KCl, 8M urea, 50 mM Tris, pH 7.5. Linear gradient was established with 750 ml starting buffer and 750 ml starting buffer made 0.4M with KCl. Flow rate: 13 ml/hr operated at room temperature; fraction size: 10 min. Effluent was monitored by absorbance at 280 nm.

Fig. 16b. SDS and SDS-urea polyacrylamide gels of T3 and T4.


a.
1.2
0.8
0.4
0
A(280nm)
|← T3 →|
|← T4 →|
0.4
0.2
0
[KCl],M
60
100
140
180
220
FRACTION NUMBER
b.
-urea
T3
I
T4
+urea
T4
T3

purified fragments T3 (27.3 mg) and T4 (22.6 mg) were 17 and 15% respectively, with reference to the amount of αTm used.

Unfortunately, the peptides in peak II were lost in the process of purification.

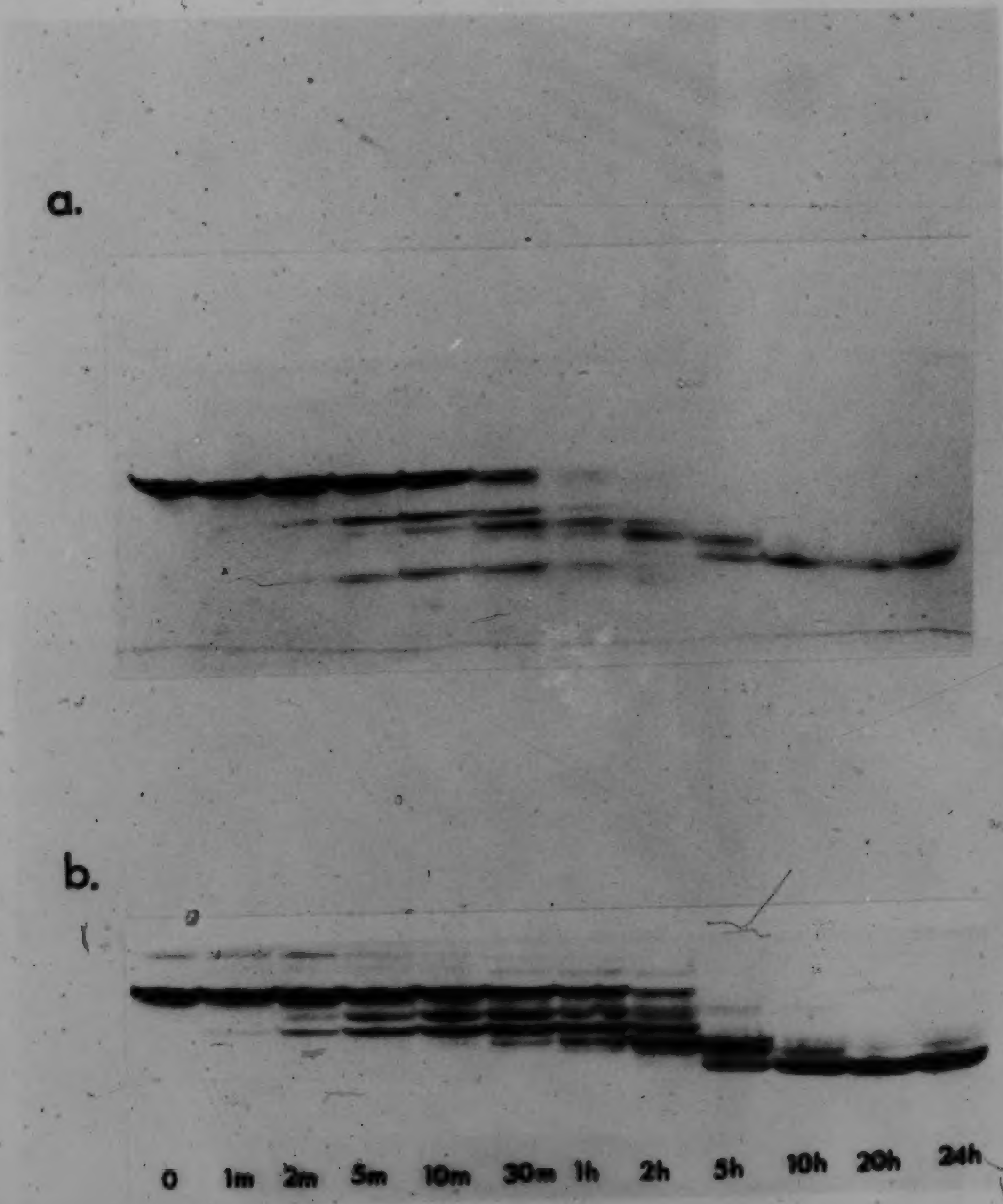
2. Digestion with Chymotrypsin [3.4.4.5]

A time course study of the chymotryptic digestion of αTm was made in the same manner as the tryptic digestion. The rate of reaction at 0°C was very slow so the enzymic reactions at an enzyme to substrate ratios of 1:200 and 1:500 at pH 8.5 were performed at 25°C. SDS and SDS-urea gels (10%) (Fig. 17) of the products of digestion at pH 8.5, 25°C and at an enzyme to substrate ratio of 1:500 revealed that two large fragments were initially formed and as the reaction time progressed, the number of peptides increased. Careful analysis of the products with respect to time suggested that the best conditions for the formation of the initial peptides were 25°C, pH 8.5 for 5 minutes at an enzyme to substrate ratio of 1:500. It is interesting that chymotrypsin required harsher conditions to hydrolyze αTm than trypsin.

Large scale preparation of the two fragments was done using the chosen conditions on an 8 mg/ml dialyzed solution of αTm (286 mg) in 50 mM NH_4HCO_3, pH 8.5. The reaction was terminated by lowering the pH to 2.0 with formic acid followed by lyophilization of the digest.

The freeze dried material was chromatographed on a

Fig. 17. Time course study of chymotryptic digestion of αTm. SDS polyacrylamide gels in the absence (a) and presence (b) of 6M urea of the products of chymotryptic digestion of αTm at 25°C, pH 8.5 at an enzyme to substrate ratio of 1:500 at different digestion times.


a.
b.
0
1m
2m
5m
10m
30m
1h
2h
5h
10h
20h
24h

CM-32 cellulose column (1.2 x 50 cm) at pH 4.0 in 8M urea. The elution profile (Fig. 18a) was constructed from the absorbances of the 2 ml fractions collected. SDS and SDS-urea gels (10%) (Fig. 18b) of the material from the peaks showed that the first peak was pure CT2 (12 mg; 10.4% yield), the lower molecular weight fragment. The second peak consisted of the higher molecular weight fragment and undigested αTm.

Separation of the large fragment, CT1, from Tm was accomplished by fractionation of the material (162 mg) from the second peak on a Sephadex QAE-A50 column (2 x 25 cm), at pH 7.5 in the presence of 8M urea (Fig. 19a and b). A yield of 8.7% for CT1 was obtained.

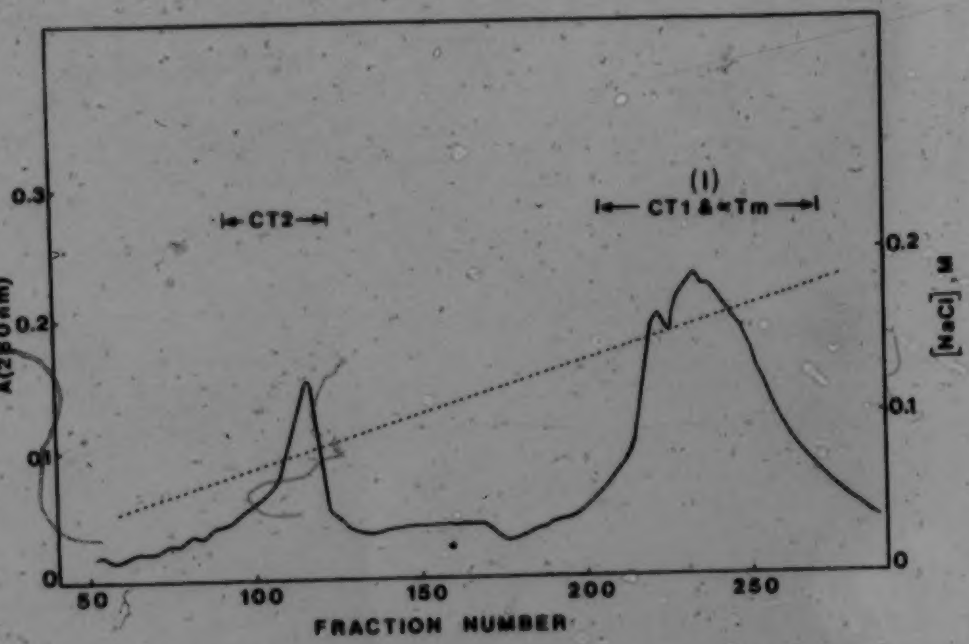
3. Digestion with Carboxypeptidase-A [3.4.2.1]

The loss of the ability of αTm to polymerize at low ionic strength could be accomplished by the cleavage of at least 4 residues from the carboxyl terminal end of αTm. Non-polymerizable Tm was prepared according to the method described by Johnson and Smillie (1977). Digestion was carried out at pH 8.0 at 34°C at an enzyme to substrate ratio of 1:200. The extent of digestion was followed by the determination of viscosity of the digest as well as the amino acid liberated as digestion progressed. The reaction was terminated by lowering the pH to 2.0 and the digest was dialyzed against 0.5% HCOOH and lyophilized. These treatments irreversibly denatured the enzyme.

Fig. 18a. Fractionation of products of chymotryptic digestion of αTm on CM-32 cellulose. Column dimensions: 1.2 x 50 cm. Starting buffer: 8M urea, 50 mM sodium formate, 0.25 mM DTT, pH 4.0. Linear gradient was established with 300 ml starting buffer and 300 ml starting buffer made 0.2M with NaCl. Flow rate: 6 ml/hr operated at room temperature; fraction size: 2 ml. Effluent was monitored by absorbance at 280 nm. Fractions were pooled as indicated.

Fig. 18b. SDS and SDS-urea polyacrylamide gels of pooled fractions.

a.


A(280nm)
0.3
0.2
0.1
0
[NaCl], M
0.2
0.1
0
50
100
150
200
250
FRACTION NUMBER
CT2
(I)
CT1 & αTm

b.

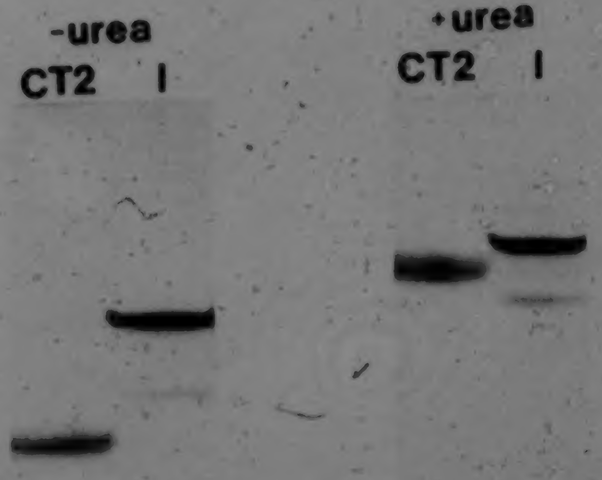
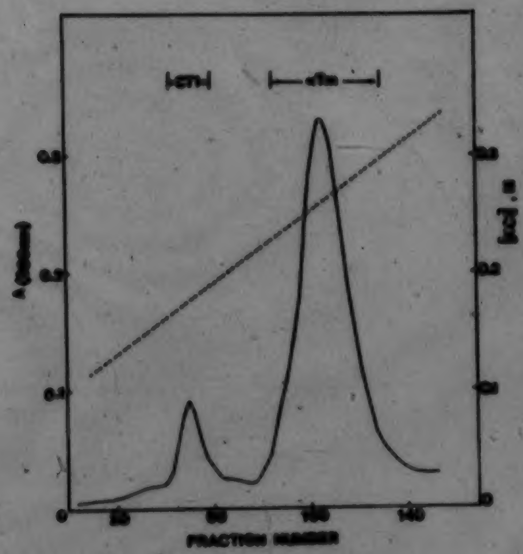

-urea
CT2
I
+urea
CT2
I

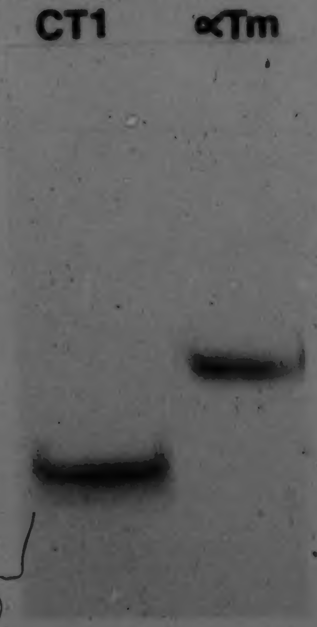
Fig. 19a. Fractionation of CT1 and αTm on Sephadex QAE-A50. Column dimensions: 2 x 25 cm. Starting buffer was 0.1M KCl, 8M urea, 50 mM Tris, pH 7.5. Linear gradient was established with 250 ml starting buffer and 250 ml starting buffer made 0.4M with KCl. Flow rate: 15 ml/hr operated at room temperature; fraction size: 2.5 ml. Effluent was monitored by absorbance at 280 nm. Fractions were pooled as indicated.

Fig. 19b. SDS polyacrylamide gels of CT1 and uncleaved αTm.

a.

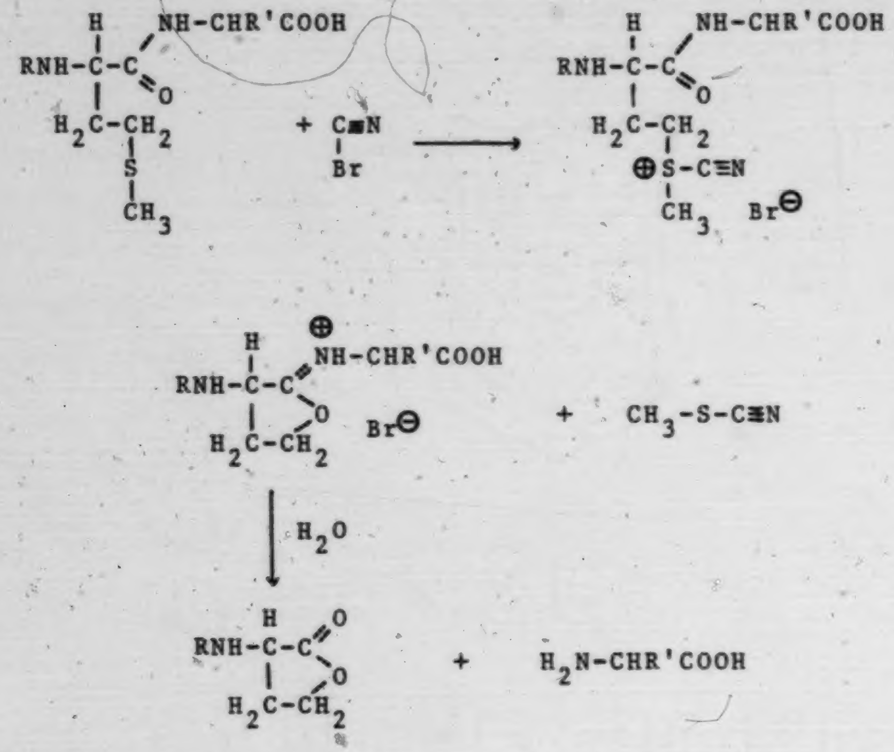

CT1
αTm
0.3
0.2
0.1
0
20
60
100
140
FRACTION NUMBER
A (280nm)
[KCl], M
0.3
0.2
0.1
0

b.


CT1
αTm

4. Reaction with CNBr

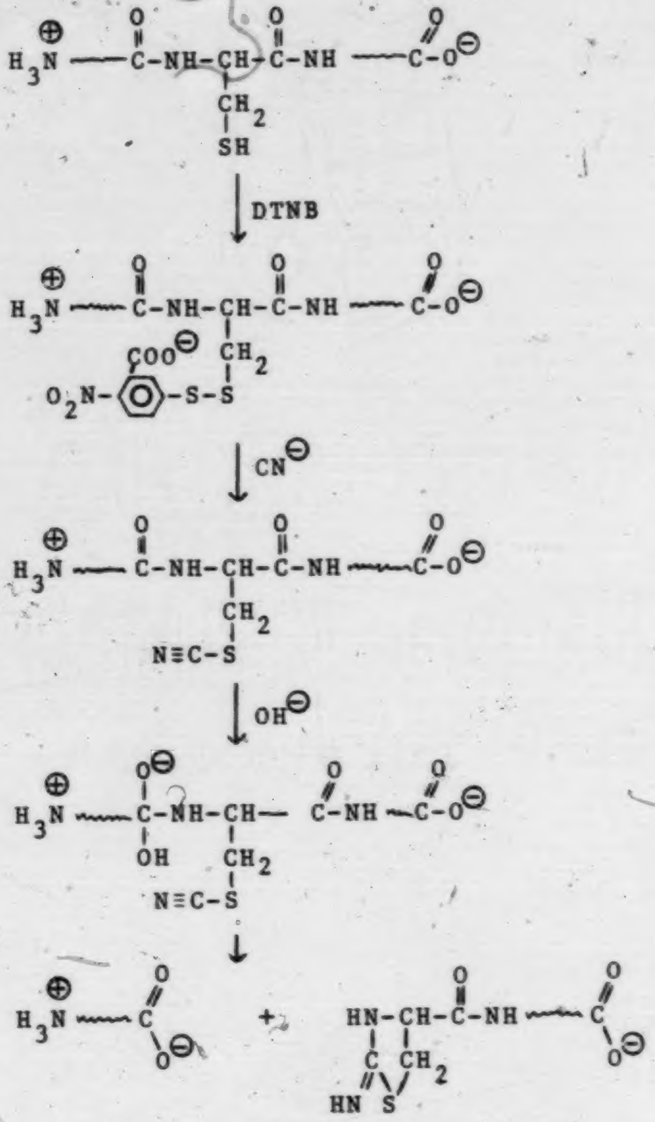
CNBr specifically hydrolyzes peptide bonds after a methionine residue as shown by the following reaction mechanism (Gross, 1967).


H
NH-CHR'COOH
RNH-C-C
O
H2C-CH2
S
CH3
+ C≡N
Br
⊕S-C≡N
Br⊖
⊕
H2O
+ CH3-S-C≡N
+ H2N-CHR'COOH

Digestion of Tm with CNBr produced two large peptides. The preparation, separation and purification of these peptides, CN1A and CN1B, were done according to the procedure of Hodges and Smillie (1973).

5. Reaction with DTNB and KCN

DTNB, 5,5'-dithiobis(2-nitrobenzoic acid), modifies the side group of cysteine and subsequent addition of KCN, potassium cyanide, results in the hydrolysis of the peptide bond on the amino terminal side of the modified cysteine. The reaction mechanism (Jacobson et al., 1973) for this reaction is shown below.



αTm has only one cysteine residue thus making this reaction very valuable for producing peptides of αTm. The two step method described by Jacobson, et al., (1973) was followed for their preparation. At the end of the reaction period, the reaction mixture was dialyzed against water extensively and lyophilized. The freeze dried material (200 mg) was applied to a CM 32 cellulose column at pH 4.0 in the presence of 8M urea. This technique separated the small fragment, Cy2, from the large fragment (Cy1) and uncleaved αTm (Fig. 20).

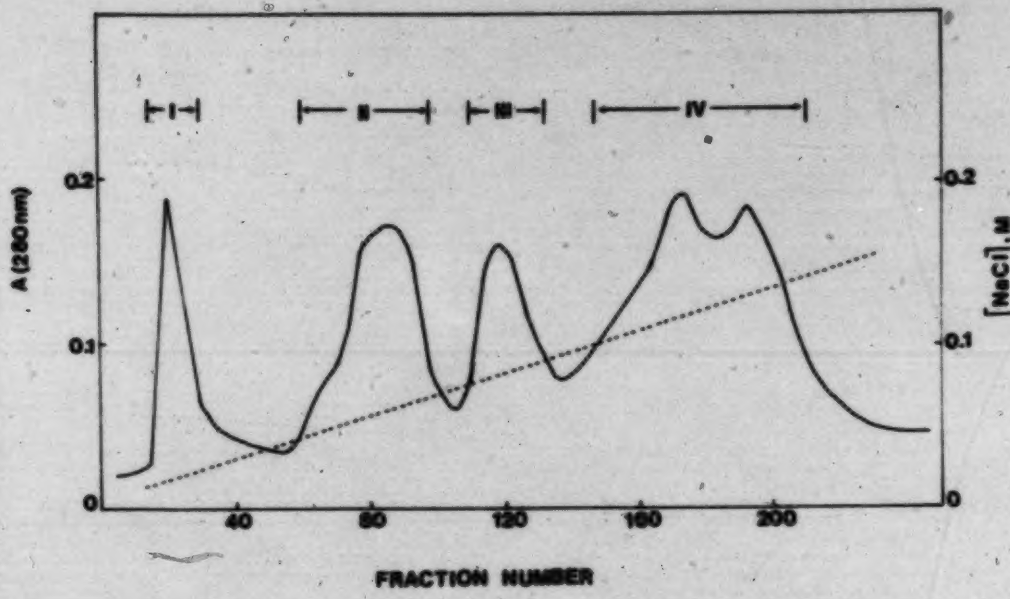
The yellow substance present in the peak I fractions may have been responsible for the high absorbance at 280 nm since no amino acids were detected when an amino acid analysis was done on the pooled, freeze dried peak I fractions. Peak II, labelled as Cy2 (17.5 mg), consisted of pure small fragment as judged from the SDS and SDS-urea gel electrophoresis (Fig. 20b). Interestingly, it was observed that peaks III and IV were both mixtures of the large fragment (Cy1) and uncleaved αTm. The reason for their elution as separated peaks is not clear.

Fractionation of the large fragment from uncleaved αTm was performed by application of 86.2 mg of Cy1 and αTm mixture to a Sephadex QAE-A50 column (2 x 25 cm) at pH 7.5 (Fig. 21a). SDS gels (Fig. 21b) of the pooled, eluted peak fractions revealed that separation of the two components was achieved. A recovery of 20 mg of Cy1 was obtained by this technique.

Fig. 20a. Separation of products of treatment of αTm with DTNB and KCN on CM-32 cellulose. Column dimensions: 1.2 x 50 cm. Starting buffer: 8M urea, 50 mM sodium formate, 0.25 mM DTT, pH 4.0. Linear gradient was established with 300 ml starting buffer and 300 ml starting buffer made 0.2M with NaCl. Flow rate: 6 ml/hr operated at room temperature; fraction size: 2 ml. Effluent was monitored by absorbance at 280 nm. Fractions were pooled as indicated.

Fig. 20b. SDS and SDS-urea polyacrylamide gels of pooled fractions.

a.


I
II
III
IV
A (280nm)
0.2
0.1
0
40
80
120
160
200
0.2
0.1
0
[NaCl], M
FRACTION NUMBER

b.

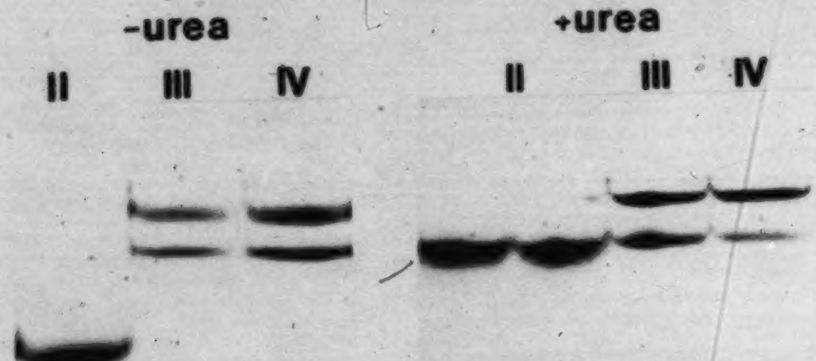
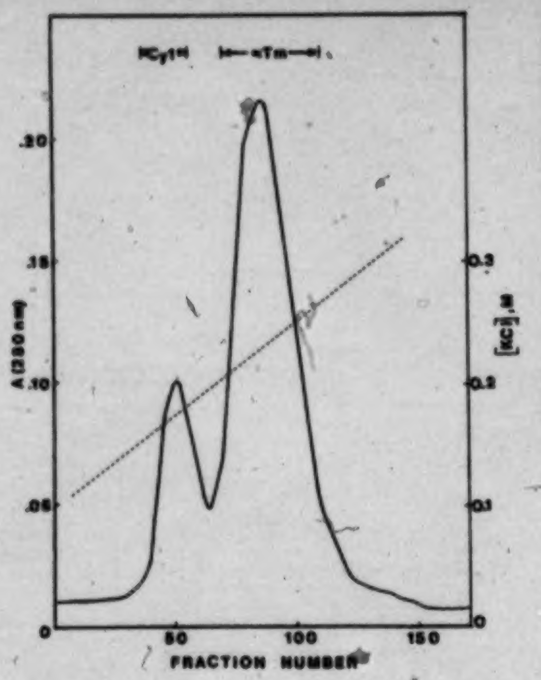

-urea
II
III
IV
+urea
II
III
IV

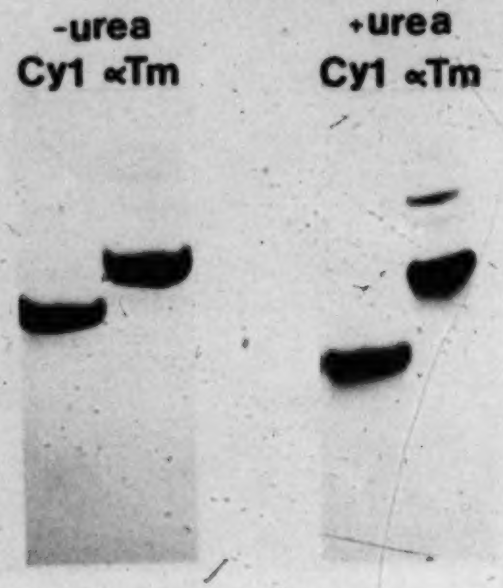
Fig. 21a. Chromatography of Cyl and αTm on Sephadex QAE-A50. Column dimensions: 2 x 25 cm. Starting buffer was 0.1M KCl, 8M urea, 50 mM Tris, pH 7.5. Linear gradient was established with 250 ml starting buffer and 250 ml starting buffer made 0.4M with KCl. Flow rate: 15 ml/hr operated at room temperature; fraction size: 2.5 ml. Effluent was monitored by absorbance at 280 nm. Fractions were pooled as indicated.

Fig. 21b. SDS and SDS-urea polyacrylamide gels of Cyl and uncleaved αTm.

a.


Cy1
αTm
.20
.15
.10
.05
0
A(280nm)
0.3
0.2
0.1
0
[KCl],M
50
100
150
FRACTION NUMBER

b.


-urea
Cy1 αTm
+urea
Cy1 αTm

This chemical cleavage is largely dependent on the presence of reduced sulfhydryl group. The αTm used for the preparation had been dialyzed in the presence of a reducing agent, mercaptoethanol, before lyophilization and was assumed to be in the reduced form. However, the low yields which were obtained, 15% for Cy1 and 26% for Cy2, may be partly explained if the αTm sulfhydryl group became oxidized after dialysis.

CHAPTER IV

CHEMICAL AND PHYSICAL CHARACTERIZATION OF αTm AND ITS FRAGMENTS

Various fragments of αTm were isolated from digestion with trypsin, T1, T2, T3, T4 and T5; with chymotrypsin, CT1 and CT2; with carboxypeptidase-A, non-polymerizable Tm; by treatment with CNBr, CN1A and CN1B; and by treatment with DTNB and KCN, Cy1 and Cy2. The purity of these fragments was established by SDS polyacrylamide gel electrophoresis in the presence and absence of urea. All of the fragments were homogeneous by this criterion except for T1, T2 and T3 which were slightly contaminated (<5%). An estimate of their molecular weights was also obtained by this technique. In order to identify these fragments, it was necessary to investigate their chemical properties.

A. CHEMICAL PROPERTIES

1. Cysteine Contents

Since αTm has only one cysteine residue at position 190, the presence or absence of this amino acid in a polypeptide is a good indicator of the origin of the fragment. The cysteine contents of the different fragments, determined as cysteic acid, (Table I) suggested that T2, T4, T5 and CT2 are derived from the COOH-terminal half of the molecule. Because CN1B was prepared from carboxymethylated αTm its cysteine content was measured as carboxymethyl cysteine.

TABLE I

Amino Acid Compositions of Fragments of αTm

	T1 1-133[a]	T2 134-284	T3 13-125	T4 183-284	T5 183-244	Cy1 1-189	Cy2 190-284	CT1 1-169	CT2 170-284
Lys	19.0 (19)[b]	18.6 (20)	14.2 (14)	12.6 (14)	8.2 (9)	26.1 (26)	13.4 (13)	24.6 (25)	14.7 (14)
His	0.1 (0)	1.6 (2)	0 (0)	0.7 (1)	0 (0)	0.9 (1)	1.0 (1)	0.9 (1)	0.8 (1)
Arg	7.9 (8)	6.2 (6)	7.2 (7)	2.9 (2)	1.4 (2)	12.0 (12)	2.5 (2)	10.3 (10)	4.4 (4)
Asp	14.6 (15)	13.9 (14)	15 (14)	10.2 (10)	4.4 (4)	18.9 (19)	10.6 (10)	18.0 (18)	11.1 (11)
Thr	3.1 (3)	4.6 (5)	3.8 (3)	4.6 (5)	2.9 (3)	3.3 (3)	5.0 (5)	3.2 (3)	4.9 (5)
Ser	5.9 (6)	8.2 (9)	5.9 (5)	6.6 (8)	4.1 (4)	8.0 (8)	7.5 (7)	6.2 (6)	7.8 (9)
Glu	31.8 (31)	36.8 (39)	31.2 (29)	24.5 (24)	15.9 (18)	47.6 (48)	22.5 (22)	42.0 (42)	29.0 (28)
Gly	1.9 (2)	1.2 (1)	1.4 (1)	1.1 (1)	1.6 (1)	3.3 (3)	1.0 (0)	2.2 (2)	1.3 (1)
Ala	19.1 (20)	15.9 (16)	17.5 (19)	10.6 (10)	5.5 (7)	26.2 (27)	9.4 (9)	24.5 (25)	11.3 (11)
Cya	0 (0)	0.9 (1)	0 (0)	1.2 (1)	1.3 (1)	0 (0)	0.2 (1)	0 (0)	0.8 (1)
Val	3.7 (4)	4.7 (5)	4 (3)	3.4 (3)	2 (2)	5.5 (6)	2.8 (3)	4.7 (5)	4.1 (4)
Met	3.2 (4)	1.9 (2)	0 (0)	0.6 (1)	0 (0)	4.8 (5)	1.1 (1)	3.9 (5)	1.3 (1)
Ile	2.8 (3)	6.8 (9)	1.2 (1)	3.2 (4)	1.2 (1)	7.7 (8)	3.8 (4)	5.5 (6)	5.7 (6)
Leu	17.0 (17)	16.0 (16)	16.0 (16)	13.0 (13)	7.0 (7)	21.0 (21)	12.0 (12)	19.0 (19)	14.0 (14)
Tyr	1.3 (1)	4.1 (5)	1.2 (1)	3.4 (4)	2.0 (2)	1.3 (2)	3.4 (4)	1.5 (2)	3.6 (4)
Phe	0 (0)	0.7 (1)	0 (0)	0.8 (1)	0.3 (1)	0 (0)	0.6 (1)	0 (0)	0.5 (1)
Molecular Weight	15,062	17,208	12,731	11,677	6,412	21,694	11,022	19,439	13,277

[a] Peptides are designated according to their position in αTm sequence (Stone, *et al.*, 1974).

[b] ( ), values obtained from sequence data.

The cysteic acid content of Cy2 is low because the sulfhydryl group of its cysteine residue has been modified in the preparation of the fragment. By the process of elimination, the remaining peptides are derived from the NH_2-terminal half of the molecule.

2. NH_2-terminal Sequences

The sequence of at least ten amino acids at the NH_2-terminal ends of the fragments was determined by automated Edman degradation. T1 and CT1 did not sequence, providing additional evidence for the assignment of these fragments to the NH_2-terminal half of the molecule. Table II lists the ten amino acids and their yields in nmoles in the order in which they were recovered during the Edman degradation. The homogeneity of the peptides was also established from the quantitative determination of the amino acid derivatives formed at each Edman degradation step.

3. COOH-terminal Sequences

A knowledge of both the NH_2- and COOH-terminal sequences of the polypeptides allows their identification since the complete sequence of the molecule is known. Penicillo carboxypeptidase-S1 and carboxypeptidase-A were employed for this purpose and the type of enzyme used was dictated by the solubility of the fragments in the conditions for optimal activity of the enzyme. Penicillo carboxypeptidase-S1, which is optimally active at pH 4.2,

TABLE II

Automated NH_2-terminal sequence Analyses of Fragments of αTm

Step of Edman degradation	Residue[a] (yield in nmoles)[b]				
	T2	T3	T4	T5	CT2
1	Ala (44)	Leu (9)	Ala (14)	Ala (53)	Val (40)
2	Gln (30)	Asp (12)	Glu (13)	Glu (39)	Ile (20)
3	Lys (31)	Lys (3)	Leu (11)	Leu (29)	Ile (36)
4	Asp (14)	Glu (9)	Ser (8)	Ser (20)	Glu (24)
5	Glu (18)	Asp (9)	Glu (13)	Glu (20)	Ser (28)
6	Glu (18)	Ala (8)	Gly (10)	Gly (17)	Asp (22)
7	Lys (29)	Leu (7)	Lys (6)	Lys (11)	Leu (13)
8	Met	Asp (8)	Cys (6)	Cys (7)	Glu (12)
9	Glu (16)	Arg (3)	Ala (7)	Ala (9)	Arg (31)
10	Ile (28)	Ala (9)	Glu (10)	Glu (13)	Ala (9)
dry weight of sample (mg)	3	2	2	2	2

---

[a] Determined by a combination of amino acid analysis after HI hydrolysis of thiazolinone derivatives, and thin layer and gas liquid chromatography of the PTH derivatives.

[b] Yields derived from amino acid analysis.

was used to sequence T1, T3, T5 and CT1. T2, T4 and CT2 were insoluble at pH 4.2. However, T2 dissolved at pH 7.8 in the absence of urea while T4 and CT2 dissolved at this pH in the presence of 4M urea. Under these conditions, carboxypeptidase-A is active and was used for the determination of the C-termini sequences of these fragments. Representative digestion profiles of some fragments using different enzymes and reaction conditions are illustrated in Figures 22, 23, and 24, and the COOH-terminal sequences of the fragments obtained from these experiments as well as the predicted sequences based on the known primary structure of αTm are presented in Table III.

4. Amino Acid Compositions

On the basis of the information gathered from the preceding experiments, the identities of the peptides were established (Fig. 25). To further substantiate these findings, the experimental amino acid compositions of the fragments were compared to their theoretical values. Table I shows good agreement in these values, providing good evidence that the assignment of the identity of the peptides was correct.

The fragments prepared by chemical cleavages at specific bonds were not subjected to the chemical tests. Their identities were deduced from knowledge of the sequence of αTm and the specificity of the reaction. This assumption was proven to be correct since the experimental

Fig. 22. Digestion of T2 with carboxypeptidase-A-DFP. The reaction was performed in 50 mM sodium phosphate, pH 7.8 buffer at an enzyme to substrate ratio of 1:10 at room temperature. Aliquots of the digest were taken at various times and the reaction was quenched by lowering the pH to 2. The amino acids released were determined in the D500 amino acid analyzer.

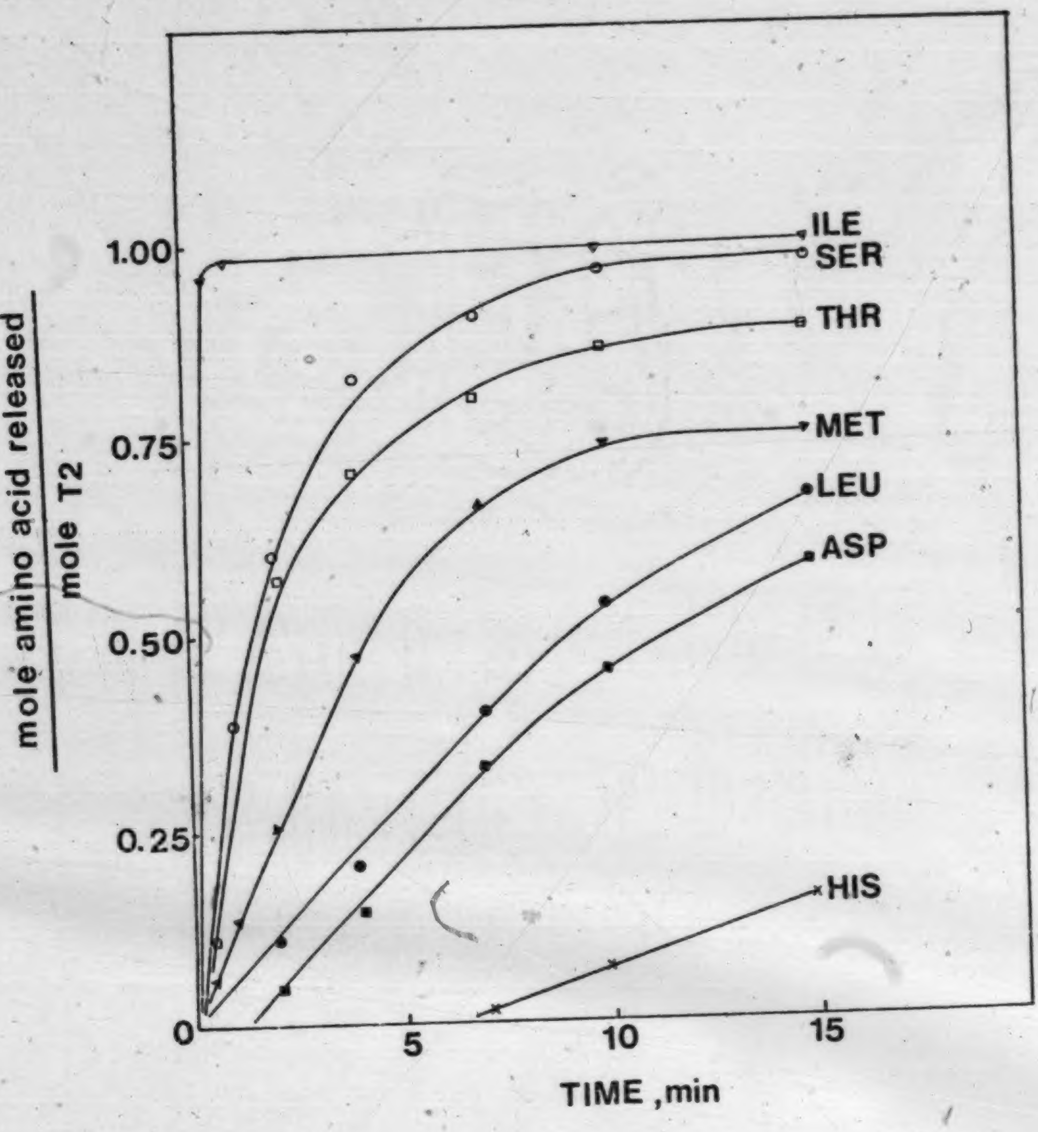

mole amino acid released / mole T2
1.00
0.75
0.50
0.25
0
5
10
15
TIME ,min
ILE
SER
THR
MET
LEU
ASP
HIS

Fig. 23. Digestion of T4 with carboxypeptidase-A-DFP in the presence of 4M urea. Same conditions were used as in Figure 22.

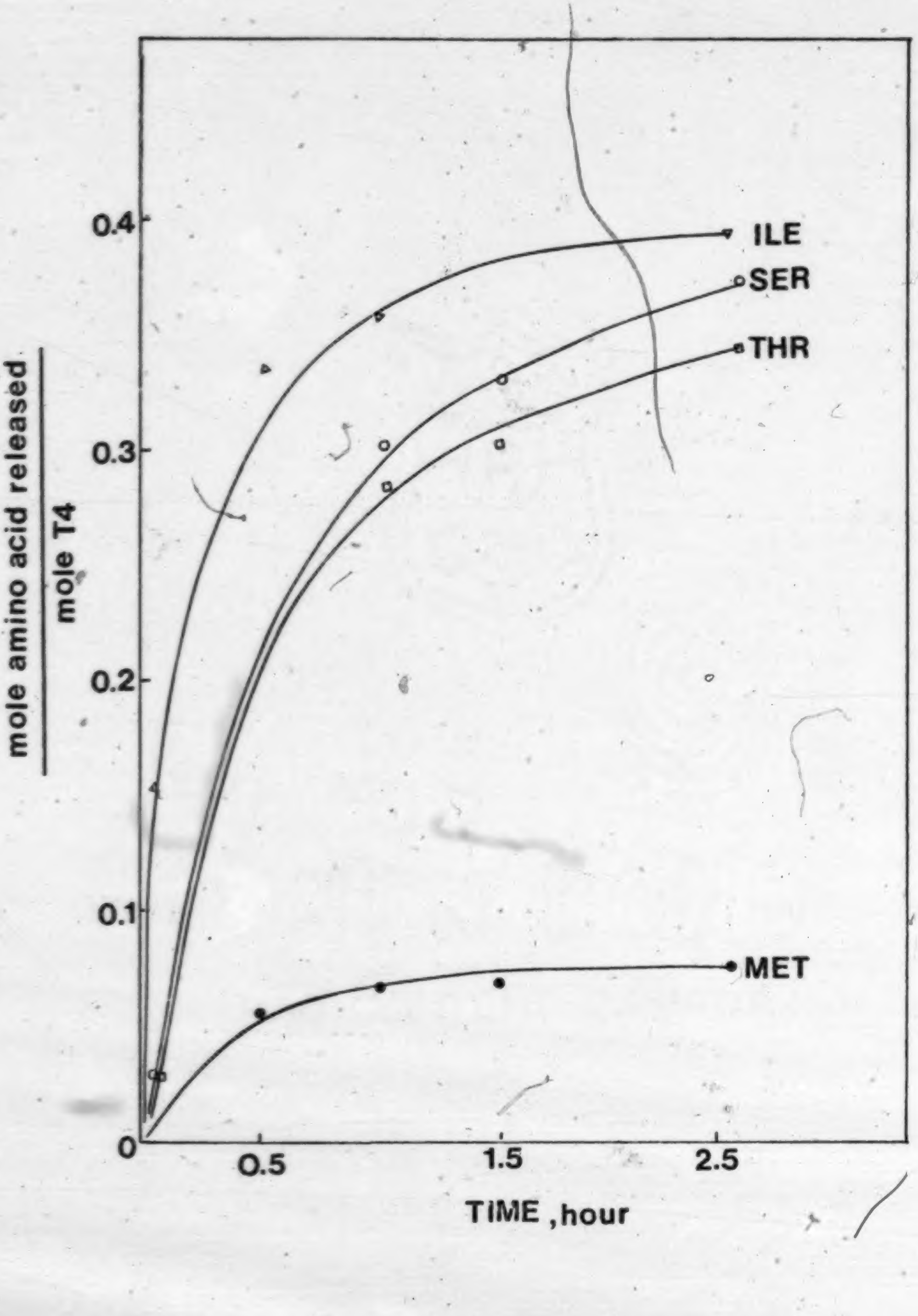

mole amino acid released / mole T4
0.4
0.3
0.2
0.1
0
0.5
1.5
2.5
TIME ,hour
ILE
SER
THR
MET

Fig. 24. Digestion of T5 with penicillocarboxypeptidase S-1. The reaction was performed in 0.2M pyridine, 0.25M HCOOH, pH 4.2 at an enzyme to substrate ratio of 1:1000 at 37°C. Aliquots of the digest were taken at various times and the reaction was terminated by heating at 100°C for 5 minutes. The amino acids released were determined on the D500 amino acid analyzer.

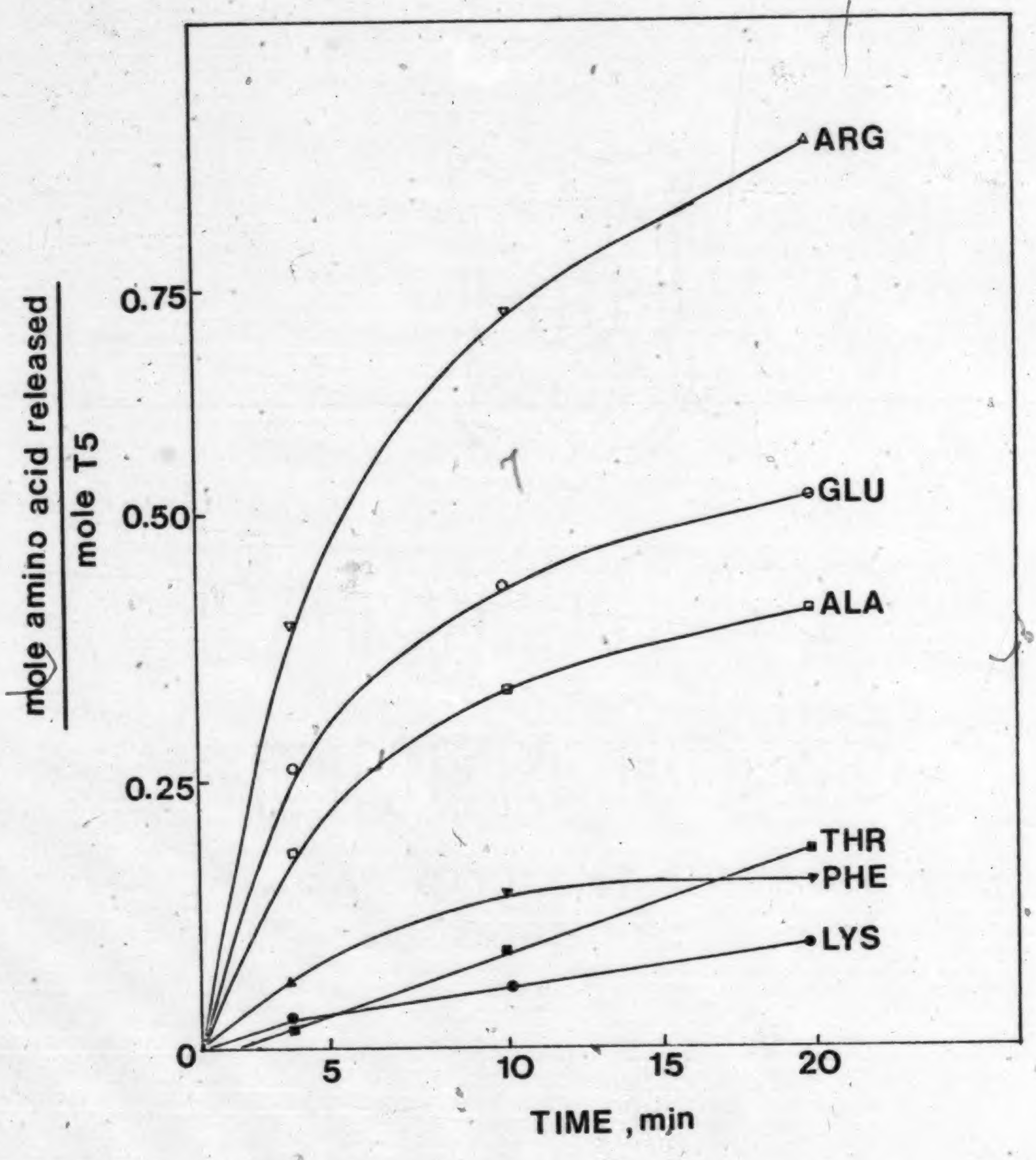

ARG
GLU
ALA
THR
PHE
LYS
0.75
0.50
0.25
0
5
10
15
20
mole amino acid released / mole T5
TIME , min

TABLE III

COOH-terminal Sequence Analysis of
Fragments of αTm

Fragments	Enzyme	Experimental[a] and Theoretical[b] Sequence
T1	Pen CP-S1	-(Gly,Ile,Val,Met,Lys,)Glu-Ser-Arg[c] -Gly-Met-Lys-Val-Ile-Glu-Ser-Arg (133)
T2	CP-A	-(Asp,Leu)Met-Thr-Ser-Ile -Ala-Leu-Asn-Asp-Met-Thr-Ser-Ile (284)
T3	Pen CP-S1	-(Asp-Lys-Ala)Ser-Glu-Arg -Lys-Ala-Ala-Asp-Glu-Ser-Glu-Arg (125)
T4	CP-A+4M urea	(Asp,Ala,Leu)Met-Thr-Ser-Ile -Ala-Leu-Asn-Asp-Met-Thr-Ser-Ile (284)
T5	Pen CP-S1	-(Asp,Leu,Lys,Thr)Phe-Ala-Glu-Arg -Thr-Arg-Ala-Glu-Phe-Ala-Glu-Arg (244)
CT1	Pen CP-S1	-(Asp,Val,Ala,Glu)Arg-Lys-Leu -Tyr-Glu-Gln-Val-Ala-Arg-Lys-Leu (169)
CT2	CP-A+4M urea	-(Ala,Glu,Met,Leu,Asp)Thr-Ser-Ile -Ala-Leu-Asn-Asp-Met-Thr-Ser-Ile (284)

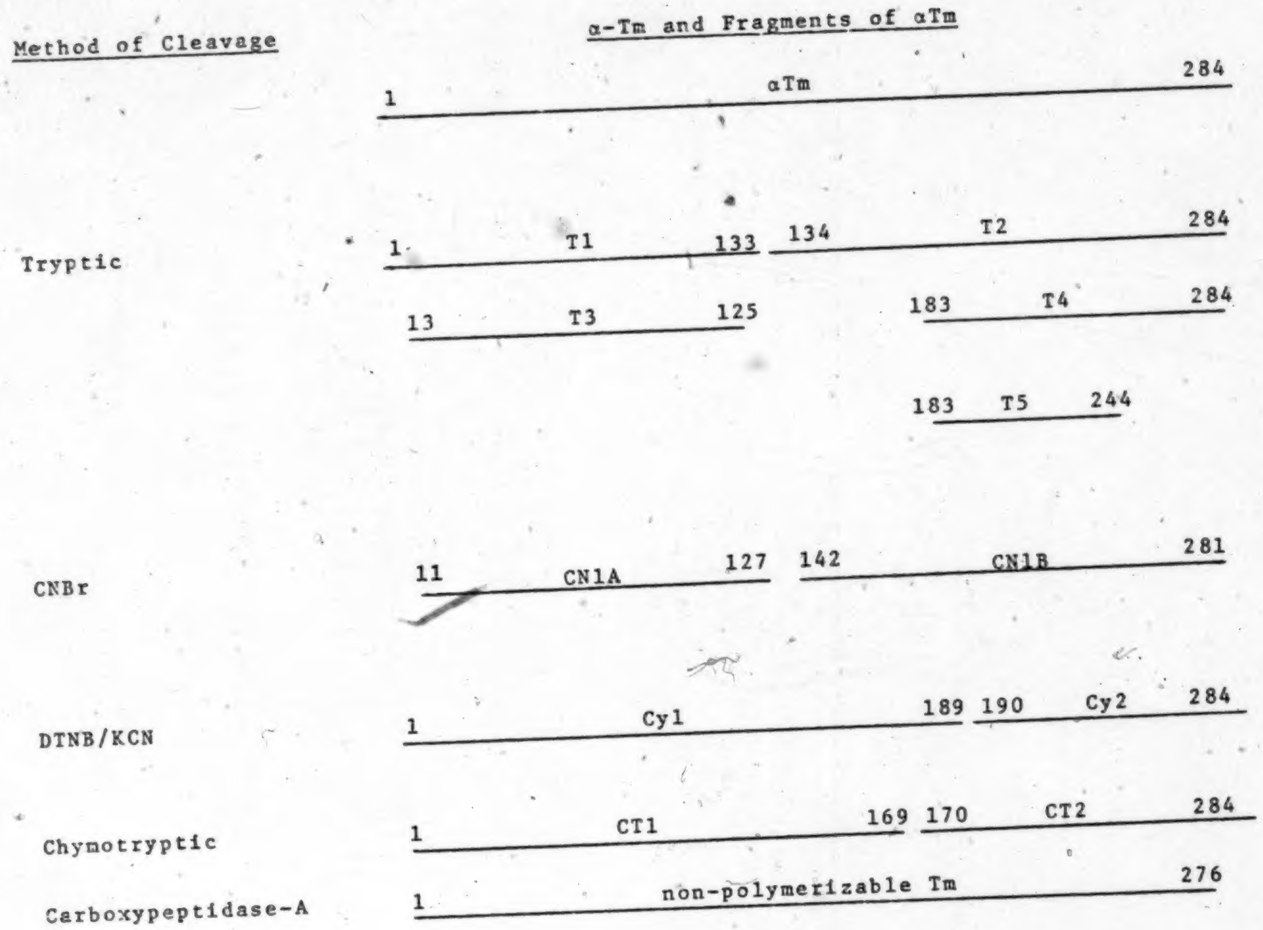
a - Upper Line

b - Lower Line

c - ←, residue determined using carboxypeptidase enzyme as indicated

Fig. 25. Fragments of α tropomyosin produced by enzymic and chemical cleavages.

9


Method of Cleavage
α-Tm and Fragments of αTm
1
αTm
284
Tryptic
1
T1
133
134
T2
284
13
T3
125
183
T4
284
183
T5
244
CNBr
11
CN1A
127
142
CN1B
281
DTNB/KCN
1
Cy1
189
190
Cy2
284
Chymotryptic
1
CT1
169
170
CT2
284
Carboxypeptidase-A
1
non-polymerizable Tm
276

amino acid compositions of these fragments agreed well with their theoretical compositions.

B. PHYSICAL PROPERTIES

The fragments of αTm were prepared with the intention of using them for interaction studies with other contractile muscle proteins to hopefully provide a fuller understanding of the biochemical properties of the intact molecule. Towards this end, it was important to elucidate to what extent the physical properties of the fragments resembled those of αTm. Since αTm has a very high helical content, this property was chosen as characteristic of the molecule. With the utilization of circular dichroism, the helical contents and stability to heat denaturation of the fragments were investigated.

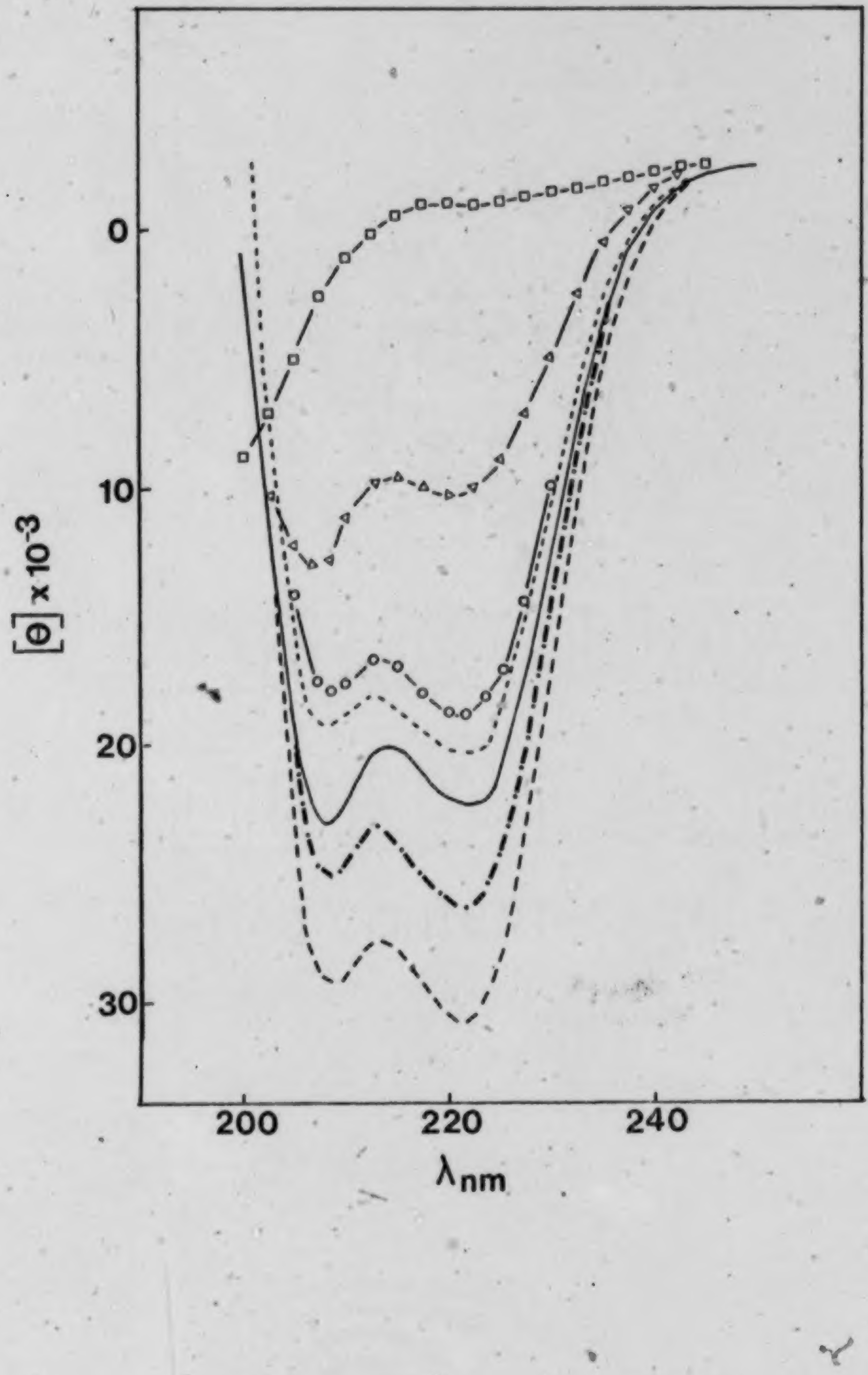
1. α Helical Contents

Ellipticities were obtained on approximately 1 mg/ml polypeptide solutions in 0.1M KCl, 1 mM DTT, 50 mM sodium phosphate, pH 7.0 buffer at various temperatures and wavelengths. The circular dichroism spectra of the fragments (Fig. 26) at 27°C from 200 to 250 nm exhibited the characteristic troughs at 208 and 221 nm of helical proteins, with the exception of T5, the shortest fragment isolated.

Using the ellipticity values obtained at 222 and 210 nm, the mean residue weight ellipticities (θ) and the α

Fig. 26. Circular dichroism spectra of various fragments of αTm at 27°C. Solvent: 0.1M KCl, 1 mM DTT, 50 mM sodium phosphate buffer, pH 7.0.

T5 (-□-), Cy2 (-Δ-), T4 (-o-), T2 (---) Cy1 (—), T3 (-·-) and T1 (— — —).


[θ] x 10⁻³
0
10
20
30
200
220
240
λnm

helical contents of the fragments were calculated with the parameters reported by Chen, et al., (1974). These authors reported the (θ) for 100% random coil protein as -2200 at 210 nm and +1580 at 222 nm; and (θ) of a helix of infinite length as -38,500 at 210 nm and -39,500 at 222 nm. Values obtained from these calculations (Table IV) revealed that all the fragments except T5 were helical to differing extents under the conditions of the experiment. This observation proved the ability of the fragments to renature to a conformation similar to αTm after being subjected to denaturing conditions such as solutions of 8M urea and 6M guanidine hydrochloride. Comparison of the helical contents of the fragments showed that those fragments derived from the NH_2-terminal half of the molecule are more helical than those originating from the COOH-terminal half of αTm.

2. Melting Temperatures

The loss of helical content of polypeptides with increasing temperature is illustrated in the circular dichroism spectra of T1 at various temperatures (Fig. 27). The melting temperature curves of αTm and its fragments were constructed from their ellipticity values at 221 nm as the temperature of the sample was increased from 10° to 70°C. The midpoint of the transition was taken to be the melting temperature and a measure of the stability of the sample to heat denaturation.

Comparison of the helical contents and melting

TABLE IV

Helical Contents and Thermal Stabilities of α-tropomyosin and its Fragments

	Residue numbers	Helical content (%)[a] at 10°C	$T_{1/2}$[b] (°C)	Average α-Parameter[c]
α-tropomyosin	1-284	92	44	1.185
NH_2-terminal fragments:				
T1	1-133	89	49	1.194
T3	13-125	89	47	1.218
Cn1A	11-127	81	47	1.202
Cy1	1-189	63	47	1.197
CT1	1-169	71	48	1.199
COOH-terminal fragments:				
T2	134-284	81	39	1.176
T4	183-284	64	33	1.160
T5	183-244	17	25	1.199
CN1B	142-281	72	29	1.130
Cy2	190-284	62	28	1.143
CT2	170-284	74	31	1.173
Non-polymerizable Tm	1-276	-	44	-

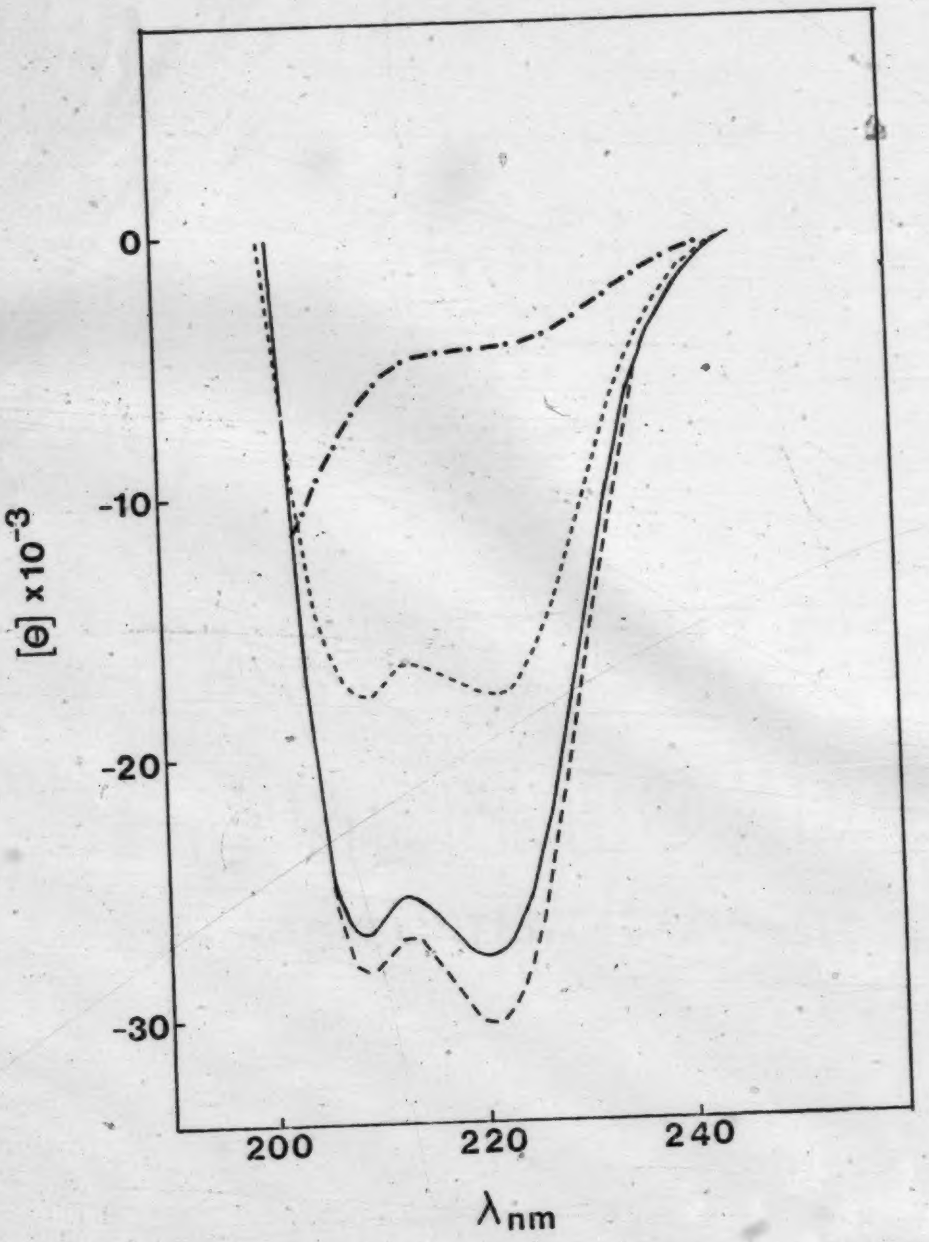
[a] Calculated from the mean residue ellipticity at 222 nm (Chen, et al., 1974; Fasman, 1976).

[b] Melting temperature.

[c] Calculated from the amino acid composition and the values given by Chou and Fasman (1974).

Fig. 27. Circular dichroism spectra of T1 at different temperatures. Solvent: 0.1M KCl, 1 mM DTT, 50 mM sodium phosphate buffer, pH 7.0.

10.3°C (— — —), 27°C and recooled to 27°C (—), 47.2°C (---) and 66°C (-·-).


0
-10
-20
-30
[Θ] x10^{-3}
200
220
240
λ$_{nm}$

temperatures of αTm in 0.1M and 1.1M KCl (Fig. 28) revealed a greater stability at high ionic strength, suggesting that hydrophobic interactions of non-polar groups in the molecule is one of the factors contributing to its stability.

The melting temperature curves of the fragments (Fig. 29) showed smooth transitions indicating a relatively uniform structure throughout the polypeptide. The process of denaturation was observed to be reversible, upon stepwise cooling, for αTm and all its fragments, except for CT2 for some reasons which are not obvious. The stability of the fragments to thermal denaturation were markedly different for fragments derived from different regions of αTm. Fragments derived from the NH_2-terminal half have higher melting temperatures than those from the COOH-terminal half of the molecule (Table IV).

The higher helical contents and thermal stabilities of the fragments from the NH_2-terminal half of the molecule as compared to those from the COOH-terminal appear to correlate with their relative contents of helix formers and breakers (Table IV) as defined by the criteria of Chou and Fasman (1974). These parameters give a number related to the likelihood of a residue forming an α helix, a β sheet or a random chain, the data being collected from the known tertiary structures of a number of crystalline proteins. This correlation is particularly true of the two cyanogen bromide fragments CN1A and CN1B where the large differences in thermal stabilities can be related to the higher content

Fig. 28. Melting temperature curves of αTm in 0.1M KCl (—) and 1.1M KCl (---) buffers containing 1 mM DTT, 50 mM sodium phosphate, pH 7.0

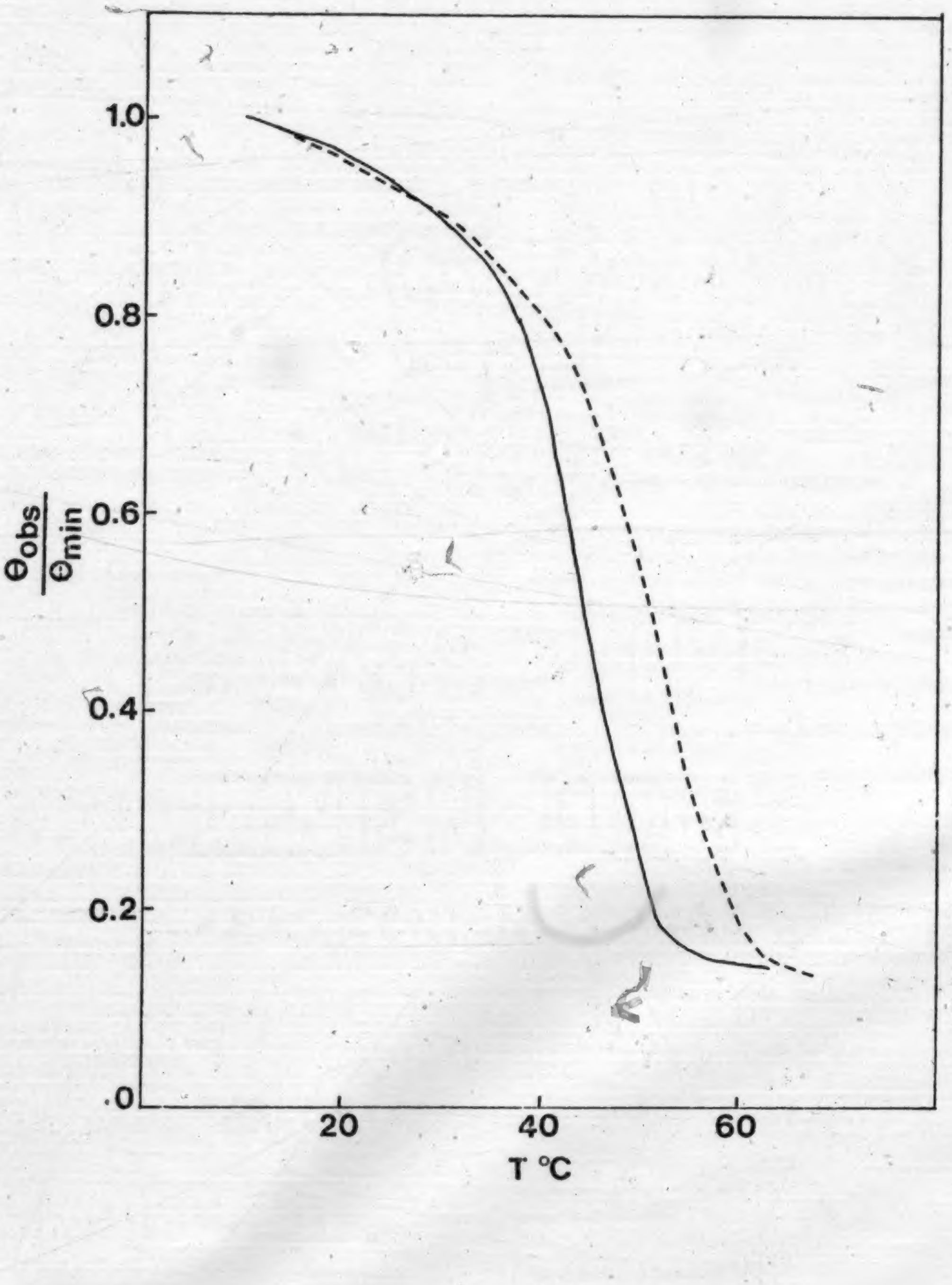
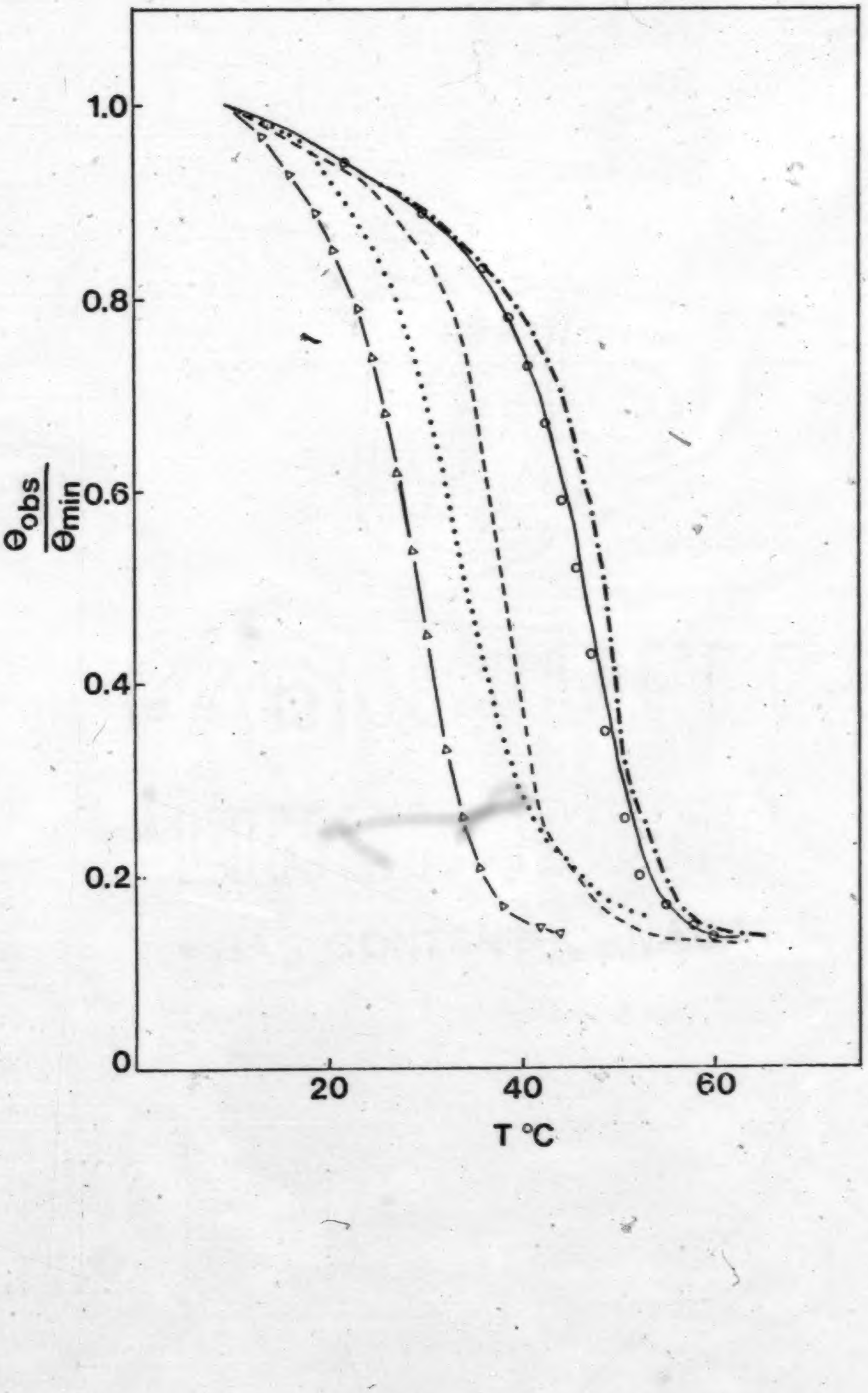

1.0
0.8
0.6
0.4
0.2
.0
$\frac{\theta_{obs}}{\theta_{min}}$
20
40
60
T °C

Fig. 29a. Melting temperature curves of various fragments of αTm in 0.1M KCl, 1 mM DTT, 50 mM sodium phosphate buffer, pH 7.0

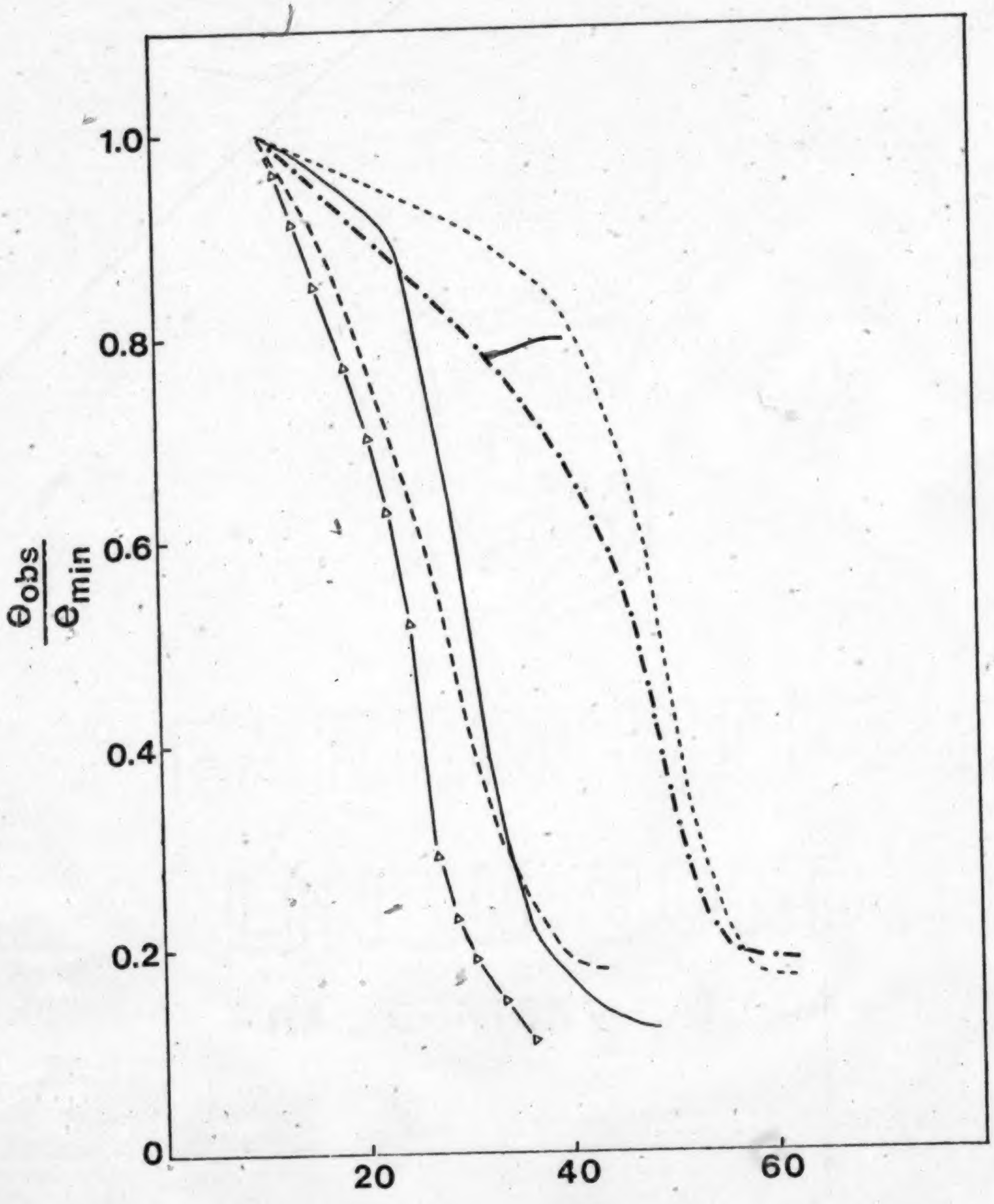
CN1B (-Δ-), T4 (·····), T2 (---), T3 (—), CN1A (o o) and T1 (-·-).



1.0
0.8
0.6
0.4
0.2
0
$\frac{\Theta_{obs}}{\Theta_{min}}$
20
40
60
T °C

Fig. 29b. Melting temperature curves of various fragments of αTm in 0.1M KCl, 1 mM DTT, 50 mM sodium phosphate buffer, pH 7.0.

T5 (-Δ-), CT2 (---), Cy2 (—), Cy1 (-·-) and CT1 (-----).


1.0
0.8
0.6
0.4
0.2
0
$\frac{\theta_{obs}}{\theta_{min}}$
20
40
60
T °C

in CN1B of such poor helical formers as tyrosine, isoleucine and serine.

C. DISCUSSION

The specificities of trypsin and chymotrypsin towards αTm provided an insight into the finer details of the structure of the molecule. Trypsin, an enzyme which hydrolyzes peptide bonds on the COOH-side of the basic residues, lysine and arginine, preferentially cleaved peptide bond 133-134 initially although αTm has 53 basic residues distributed throughout its sequence. This mode of action of the enzyme indicated the presence of a less ordered structure in this region than in the remainder of the coiled coil of αTm. Inspection of the sequence around this region revealed the occurrence of an aspartic acid residue in the hydrophobic core position 137. This is the only core position in the structure of αTm where aspartic acid is found although other charged residues such as glutamic acid and lysine do occasionally occur in these positions. In the case of the latter residues however, model building studies have demonstrated that their side chains are sufficiently long to permit their charged amino or carboxyl moiety to extend outwards to the solvent. On the other hand, aspartic acid because of its shorter side chain length, can be expected to have a more disruptive effect on the stability of the coiled coil in its immediate environment. Its presence at the core position 137 may therefore be responsible for the initial rapid cleavage of the αTm molecule by trypsin at arginine 133.

This proposal is substantiated by the recent observation of Philips and Cohen (personal communication) that the region around residue 134 is one of the cross-over points of the strands in Tm crystals which have been previously alluded to as possibly containing low helical content by Caspar, et al., (1969).

Prolonged exposure of αTm to trypsin at 0°C produced the large fragments T3, T4 and T5, which are resistant to further action of the enzyme in spite of their high basic amino acid contents. Similar trypsin resistant fragments have been described by other investigators.

Ueno and Ooi (1977) isolated two large polypeptides, s and p, after digestion of αTm with trypsin at 0°C for three days. They tentatively identified s and p fragments as polypeptides 13-149 and/or 128 and 183-284 respectively, which are the same as the T3 and T4 fragments isolated in this study.

Gorecka and Drabikowski (1977) observed the formation of two bands with molecular weights 12,000 and 17,000 daltons in SDS polyacrylamide gels after twenty minutes of digestion of Tm with trypsin at 20°C at an enzyme to substrate ratio of 1:1000. The authors did not make definite conclusions regarding the origin of the fragments because of the complication introduced by their use of unfractionated Tm which is a mixture of α and β chains. However, since the α form is dominant, the tentative assignment was made that the 17,000 dalton fragment was derived from the NH_2-terminal part of the molecule and the 12,000 dalton fragment from the COOH-terminal region. The latter fragment may correspond to T4 because of the similarity of their molecular weights but no NH_2-terminal tryptic fragment with a molecular weight of about 17,000 daltons was isolated in the present study. However, the possibility exists that T1 or T3 exhibit a higher apparent molecular weight in SDS polyacrylamide gels.

On the other hand, the fragments, TTC-A and B isolated by Eckard and Cowgill from tryptic digestion of Tm at 0°C, pH 8.5 for 24 hours at an enzyme to substrate ratio of 1:100 exhibited different chemical properties from the tryptic fragments prepared in this study. Mainly on the basis of the results obtained from carboxypeptidase-A digestion of the fragments and their histidine contents, the authors tentatively assigned the origin of their fragments. Upon carboxypeptidase-A digestion, only a very small amount of histidine was released from TTC-A whereas isoleucine, serine and threonine were liberated from TTC-B. No explanation was offered for the former observation and the interpretation of the latter result was that TTC-B was derived from the COOH-terminal region of the molecule since the C-terminus is the only part of αTm which has a sequence (-Asp-Met-Thr-Ser-Ile) compatible with the results. Since the amino acid analysis of the fragments showed that both fragments contained histidine, it was proposed that TTC-A was derived from the central one-third of the molecule since αTm has only two histidine residues, at positions 153 and 276. If the assignment of the origin of the peptides were correct, each fragment, which is about 100 residues long, should have one histidine residue. The reported experimental histidine contents of fragments TTC-A and B, 1.5 and 0.4 moles/100 residues respectively, are clearly inadequate evidence for the assignment of these fragments. The amino acid composition of TTC-A and B do not correspond to those of the tryptic fragments isolated in this study. These differences may arise from the fact that Eckard and Cowgill used unfractionated Tm whereas pure αTm was used in this study. Another possibility that could account for these differences are the presence of impurities but since no photographs of the SDS-polyacrylamide gels of the fragments were published, the homogeneity of their fragments cannot be judged.

Eckard and Cowgill further suggested that TTC-A and TTC-B were products of random attacks of trypsin at different sites on Tm. This was based on the multiple bands observed in SDS polyacrylamide gel at short digestion time (5 minutes) and the reduction to two bands at prolonged digestion time. Although the same results were observed in this study, further examination of the digestion products at shorter digestion times than 5 minutes revealed the initial production of two large fragments as the result of a specific single bond cleavage of αTm.

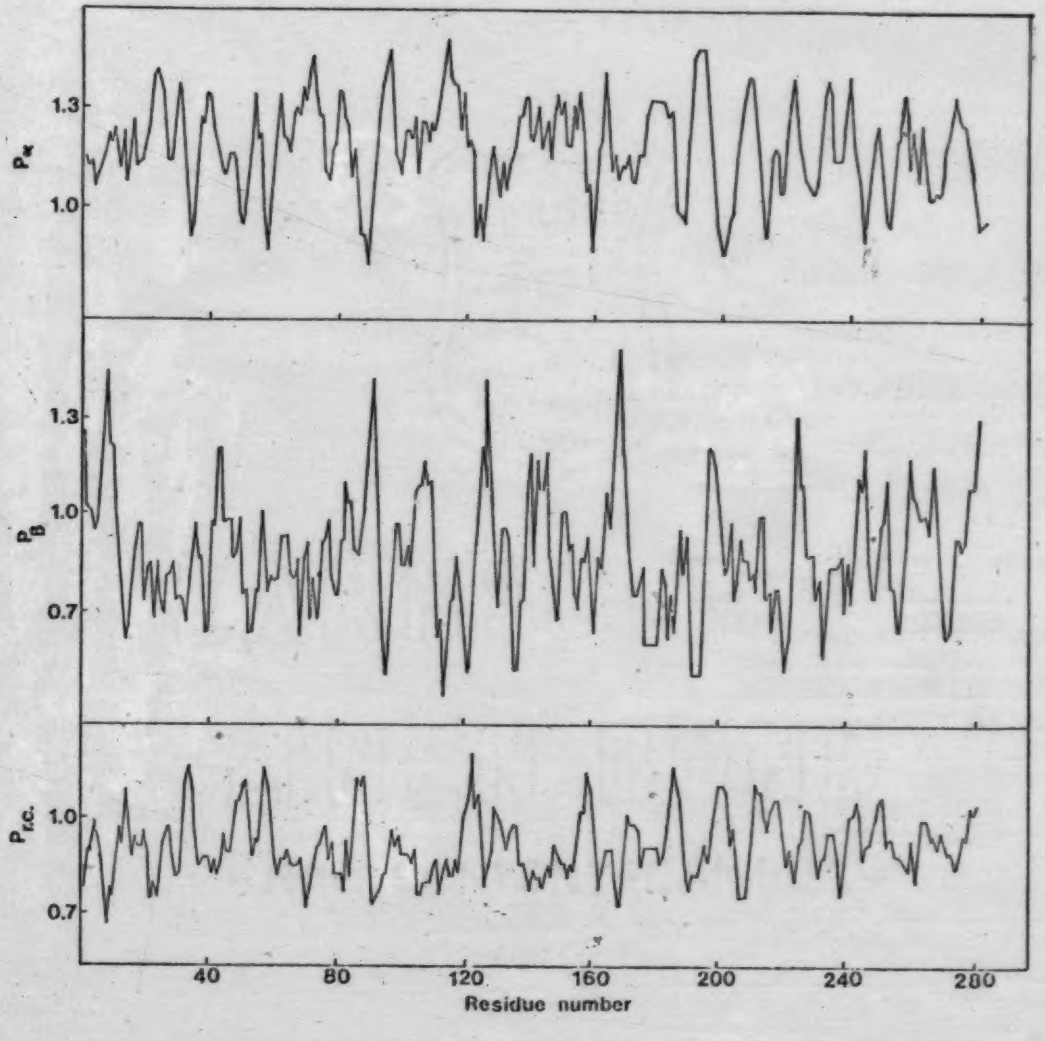
Similarly chymotrypsin, an enzyme which preferentially hydrolyzes the peptide bonds on the COOH-side of bulky hydrophobic amino acids initially cleaved peptide bond 169-170 although αTm has a high content of leucine, isoleucine and valine distributed throughout the entire molecule. There is no obvious disruption of the coiled coil around residue 169 but it may be significant that residue 169-173 (-Leu-Val-Ile-Ile-) is the only part of the molecule where four bulky hydrophobic residues occur consecutively. In most cases these residues are surrounded by charged amino acids in the molecule which may render them unfavorable to the action of the enzyme. This may partly explain the failure of the enzyme to hydrolyze peptide bonds of isoleucine 130 and 143 which are in the vicinity of residue 137 and thus presumably situated in a less helical region.

The isolation of the tryptic resistant fragments indicated that regions 13-125 and 183-284 are more ordered than the N-terminus and central portion of the molecule. These fragments were presumably formed by hydrolyzing away regions of less ordered structure from the initially formed T1 and T2 fragments. Although there is no obvious explanation from an examination of the amino acid sequence for a relative instability of the central region of the molecule, the present results do indicate that this is the case.

Detailed analyses of the primary structure of αTm were undertaken in attempts to correlate features of its structure with the experimental evidence suggesting the presence of less ordered regions and the greater stability of the NH_2-terminal half compared with the COOH-terminal half of the molecule. Three factors were considered in these analyses: (1) the formation of α helical structures is favored by the presence of certain amino acids; (2) the Tm coiled coil structure is largely maintained by the hydrophobic interactions of the core amino acids; and (3) this structure is further stabilized by ionic forces exerted by the charged groups of amino acids at positions e and g.

A computer program was written by Paul Pearlstone to calculate the mean of the conformational parameters (α, β or random coil) reported by Chou and Fasman (1974), over spans 4, 6, 8, 10, 12 and 14 amino acid residues centered at every peptide bond throughout the sequence of αTm. The values were plotted against the positions in the sequence of the molecule to detect regions less likely to be α helical. It was observed from plots of the mean of α, β and random coil parameter over a span of four amino acid residues (Fig. 30), that the α helix factor is consistently larger than the β-sheet and random coil factors. In some cases, the various conformational parameters were similar to one another but this does not necessarily infer a break in the α helix. In these instances, it is noticeable that the magnitude of the α helix factor is particularly high immediately on either side of these regions and this

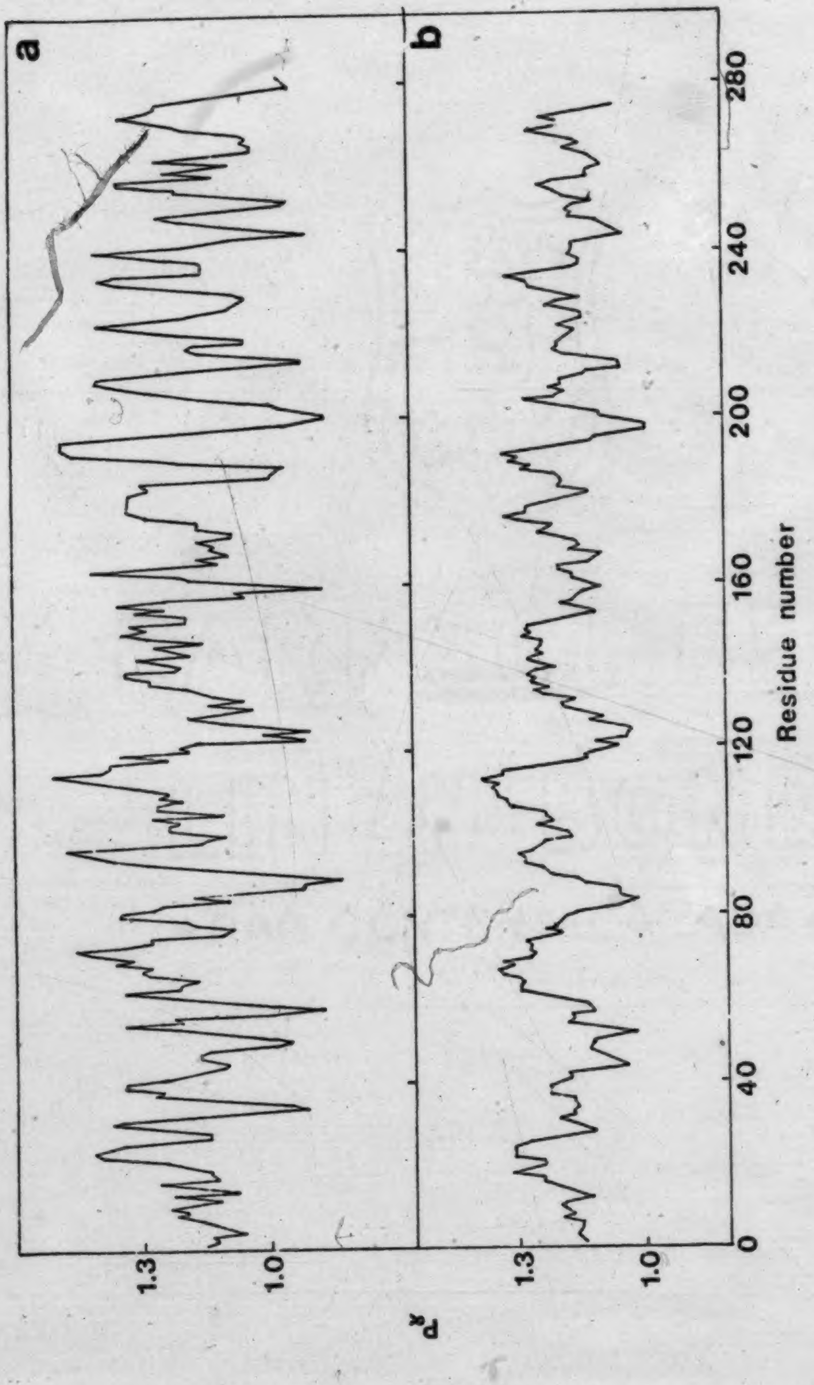
Fig. 30. Conformational parameters (a, α; b, β; c, random coil) at different positions on the sequence of αTm. The mean of the conformational parameters, reported by Chou & Fasman (1974), over a span of 4 amino acid residues centered at every peptide bond throughout the sequence of αTm were calculated and plotted against their position in the sequence of the molecule.


Pα
1.3
1.0
Pβ
1.3
1.0
0.7
Pr.c.
1.0
0.7
40
80
120
160
200
240
280
Residue number

may stabilize the latter sufficiently for the helical structure to be maintained over the entire length. This is illustrated on the plot of the mean of α parameter over a span of 10 amino acid residues (Fig. 31) where the troughs observed in Fig. 30a became shallow. The only region where the molecule may be non helical or at least distorted in some manner is at the N- and C-termini of the molecule. The same observations have been made by Parry (1975) from a similar study. Although the values of the troughs in Fig. 31 are close to or over 1.0 which means they are helical according to Chou and Fasman rules, they indicate regions of lower helical contents relative to the other parts of the molecule. However, these regions are almost evenly distributed throughout the molecule and thus, do not explain the greater stability of the NH_2-terminal half of αTm. There is no correlation between the results of this analysis and the actions of the enzymes since their preferential initial sites of cleavages, 133-134 and 169-170, occur in regions with a relatively high average α parameter. The failure of this method to detect regions that are indicated to be less helical by the enzymes could be due to the fact that the conformational parameters were obtained from globular proteins (Chou & Fasman, 1974) and may not be very effective in predicting the conformation of coiled coil structures.

As discussed previously the core positions a and d (corresponding to series I and II residues) in the sequence

Fig. 31. Comparison of the conformational parameters (α) at different positions on the sequence of αTm calculated as a mean over a span of 4 amino acid residues (a) and 10 amino acid residues (b).


a
b
P_α
1.3
1.0
1.3
1.0
0
40
80
120
160
200
240
280
Residue number

of αTm are largely occupied by non-polar amino acid residues. However, in a number of positions throughout the sequence these positions are occupied by polar or ionic residues. It has been previously suggested that their occurrence in these positions could lead to a local destabilization of the coiled coil structure (Hodges, et al., 1972). Examination of the sequence revealed that some of these amino acids occur in clusters at the N-terminus (Lys 15 and 29, and Ser 36), the central part of the molecule (Ser 123, Asp 137 and Gln 144) and about 1/4 from the C-terminus (Lys 214 and 221, and Glu 218). The combined effects of these amino acids may be sufficient to locally disrupt the α helix making it susceptible to the action of the enzyme as in the case of the formation of T3 from T1 where some amino acids from the N- and C-termini of T1 were hydrolyzed by trypsin. However, this line of argument does not apply to the formation of T4 and T5 and the initial site of cleavage of chymotrypsin. Thus another factor which could disrupt the structure was considered.

In the coiled coil, with the helices running in the same direction, position e in one helix is in a spatial arrangement to interact with position g in the preceding heptapeptide of the second helix (Fig. 7). In αTm, high proportions of the basic residues occupy the g position while acidic residues occupy the e position thus stabilizing the structure by ionic interactions. The occurrence of amino acids of the same charges in these positions would be predicted to lead to repulsion and disruption of the coiled coil. Inspection of the sequence of αTm showed that there are some acidic amino acids in the g position and basic amino acids in the e position. However, in most cases, these amino acids are paired with uncharged residues except for three positions, lys 7 and 12, asp 28 and glu 33, and asp 175 and glu 180. The disruptive effects of the first and last pairs of amino acids on the coiled coil was manifested by the digestion by trypsin of the N-terminus and central part of αTm and may also explain the preference for initial cleavage of chymotrypsin at Leu 169. However, the effects of Asp 28 and Glu 33 were not evident.

In a recent publication, Lehrer (1978) demonstrated the instability of the region around Cys-190. Addition of guanidine hydrochloride (GuHCl) to reduced Tm to a concentration between 2 and 3M GuHCl, completely dissociated the molecule (Pont & Woods, 1971). However, determination of the ability of an oxidizing reagent, NbS_2, to cross-link the sulfhydryl groups of Cys-190 residues of the two chains of αTm as a function of increasing concentration of GuHCl, showed that at 1.5M GuHCl disulfide formation was lost even before the molecule completely dissociated (Lehrer, 1978). The instability of this region is not obvious from the sequence analyses.

The results obtained from the analyses described are not sufficient to provide a clear picture of the structure of αTm as shown by the lack of good correlation between the theoretical and experimental observations presented. The local disruptions in the α-helix detected by circular dichroism, the enzymes and NbS_2 may be due to additive effects of several subtle disruptions which are beyond the sensitivity of detection of the methods of analysis employed.

CHAPTER V

INTERACTION STUDIES

A. INTERACTIONS WITH Tn AND ITS COMPONENTS

A better understanding of the complex mechanism of muscle contraction requires a full knowledge of the nature of interaction between the contractile proteins. A number of studies carried out to delineate the binding site of Tn on Tm have resulted in the hypothesis that the interaction site is about one-third of the distance from the C-terminus of the Tm molecule. This proposal evolved from studies on ordered aggregates of Tm and statistical analyses of the primary sequence of the molecule. As yet, no convincing direct experimental evidence has been reported to prove or disprove this hypothesis.

This study was undertaken in the hope that more direct experimental evidence for the binding site of Tn on Tm could be obtained. It was also anticipated that the results could provide information on the nature of the interactions of Tm with F-actin. Various large fragments of αTm were prepared and their binding to the other thin filament proteins was investigated. In the case of the binding of Tm to Tn, the obvious choice of material for these interaction studies was the TnT component. However, its insolubility in solutions of physiological ionic strength severely limited its use. Since the formation of a complex between TnC and TnT renders the latter soluble, the TnCT complex was used in some experiments. While this study was in progress, Jackson, et al., (1975) reported that a CNBr fragment of TnT, CB1, comprises the Tm binding region and unlike TnT, is very soluble at low ionic strength. In this study Tn, TnT, TnCT and CB1 were used whenever possible and appropriate.

1. Rabbit Skeletal αTm and its Fragments

(a) Ultracentrifugation

Sedimentation velocity centrifugation is a useful tool for demonstrating interaction between two proteins. Complex formation is indicated by the appearance of a peak with a sedimentation constant different from its components. This technique was utilized to investigate the interaction between TnCT and αTm, T1 or T2 at different ionic strengths.

The sedimentation velocity patterns of mixtures of TnCT and αTm in buffers containing either 0.15M (Fig. 32c) or 0.5M KCl displayed two peaks. The slow moving peak was attributed to excess αTm because of the similarity of its sedimentation constant with that of αTm sedimented alone (Table V), while the other peak was attributed to a complex between αTm and TnCT since it had a relatively high sedimentation constant and exhibited the characteristic hypersharp peak of a Tm-Tn complex (Hartshorne & Mueller, 1967). Experiments done in the presence of 1.0M KCl did not show any indication of complex formation suggesting that the binding between the two components must be stabilized, at

Fig. 32. Ultracentrifuge analysis of the interaction of TnCT with αTm. The run was made at 20°C, rotor speed of 60,000 rpm and bar angle of 65°. Sedimentation is from left to right. Photographs were taken at intervals of 16 min. The solvent contained 0.15M KCl, 1 mM DTT, 0.01 mM $CaCl_2$, 10 mM imidazole pH 7.0. a, Tm (5.5 mg/ml); b, TnCT (3 mg each of TnT and TnC per ml, 1:2 molar ratio); and c, mixture of TnCT and Tm (3.6 mg TnCT and 3.3 mg Tm per ml).

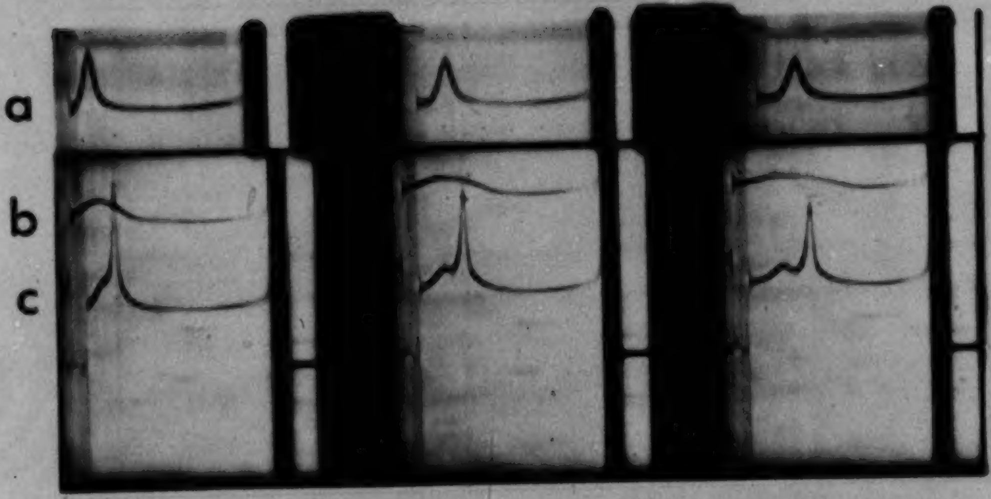

a
b
c

TABLE V

Sedimentation Coefficients of αTm, T1, T2, TnCT and their Complexes at Different Ionic Strengths

	$S_{20,w}$[b]		
	0.15M KCl[a]	0.5M KCl[a]	1.0M KCl[a]
αTm	2.5	2.4	2.5
TnCT	2.9	3.1	2.9
αTm + TnCT	2.5, 4.7	2.7, 4.1	2.5
T1	1.9		
T1 + TnCT	1.9, 5.1		
T2	2.3		
T2 + TnCT	4.7		

[a] All solutions also contained 1 mM DTT, 0.01 mM $CaCl_2$, 10 mM imidazole pH 7.0.

[b] Results from single experiments.

least in part, by electrostatic forces. T1 and T2 were also observed to form complexes with TnCT.

Although this technique detected complex formation between the proteins examined, the rapid spreading of the boundaries, particularly for TnCT (Fig. 32b), made calculation of the sedimentation constant difficult and inaccurate, and the identification of new boundaries ambiguous. TnT was not used instead of TnCT because of its insolubility in 0.15M KCl. Thus a search for a better technique was made.

(b) Polyacrylamide Gel Electrophoresis

Jackson, *et al.*, (1975) have demonstrated the interaction between TnCT and Tm by polyacrylamide gel electrophoresis in the absence of denaturing agent. A mixture of Tm and TnCT remained at the origin while Tm when run by itself entered the gel and TnCT did not. In this work, similar conditions were employed to study the binding of TnCT and CB1 with αTm and its fragments except for the incorporation of 40% glycerol in the gel which enabled TnCT to enter the gel and form a distinct band.

TnCT complex was initially prepared by mixing the components at equimolar ratio in 0.15M KCl, 1 mM DTT, 0.01 mM $CaCl_2$, 10 mM imidazole pH 7.0, but when the mixture was applied to the gel, it resolved into a TnCT band and an excess TnC band. Under the conditions of the experiment, TnT remained at the origin. Increasing amounts of TnT were added to TnC until the TnC band disappeared. At this point, the molar ratio of TnC to TnT was 1:3. These calculations were based on the extinction coefficient values $E^{1\%}_{280\text{ nm, 1 cm}}$ = 5.0 for TnT (Wilkinson, 1974) and $E^{1\%}_{277.5\text{ nm, 1 cm}}$ = 2.3 for TnC (Murray & Kay, 1972). Electrophoresis of an equimolar mixture of TnCT and αTm on a benign gel with an electrode buffer, 20 mM Tris, 125 mM glycine pH 8.6, revealed the presence of a band of slower electrophoretic mobility than the components of the mixture, indicating the formation of a complex between αTm and TnCT (Fig. 33a). Similar results were obtained using CB1 instead of TnCT (Fig. 33b).

When T1 and T2 were mixed with either TnCT or CB1 and assayed for binding, both fragments were observed to complex with both TnCT and CB1 (Fig. 34). The molar ratios of the components of the mixtures were varied and the volumes of the samples applied to the gel were calculated such that the amount of TnCT or CB1 was kept constant. Fig. 34a shows that as the concentration of T1 or T2 in the mixtures was increased, the intensity of the complex band increased while the TnCT band decreased and finally disappeared at a molar ratio of 3 fragments to 1 TnCT.

To prove that the new slower moving band was a complex of CB1 and the αTm fragments, the band was cut out and rerun in an SDS-urea polyacrylamide gel. The band was soaked in a small amount of electrode buffer containing urea and dialyzed for about 36 hours against the same buffer to dissociate the complex. The treated "complex band" was run

Figs. 33,34, 36-39.

Polyacrylamide gel electrophoresis (PAGE) of αTm, it fragments, TnCT, CB1 and mixtures of αTm or its fragments and TnCT or CB1. The molar ratios of the components of the mixtures, in 0.15M KCl, 1 mM DTT, 0.01 mM $CaCl_2$, 10 mM imidazole, pH 7.0, were varied. The mixtures were incubated at 4°C for 24 hours and made 40% with respect to glycerol just before application to the gel. Electrophoresis was performed on 6% (w/v) slab gels containing cyanogum 41, 40% (v/v) glycerol, 20 mM Tris, 125 mM glycine, pH 8.6 buffer. The electrode buffer was 0.1M KCl, 1 mM $CaCl_2$, 20 mM Tris, 125 mM glycine, pH 8.6. The experiment was carried out at 60 mA for 11 hours. The gels were stained with Coomassie Blue.

Fig. 33a. Interaction of αTm with TnCT

(1) TnCT
(2) TnCT + αTm (1:3)
(3) αTm

b. Interaction of αTm with CB1

(1) Tm
(2) αTm + CB1 (2:1)
(3) αTm + CB1 (1:1)
(4) αTm + CB1 (0.5:1)
(5) CB1

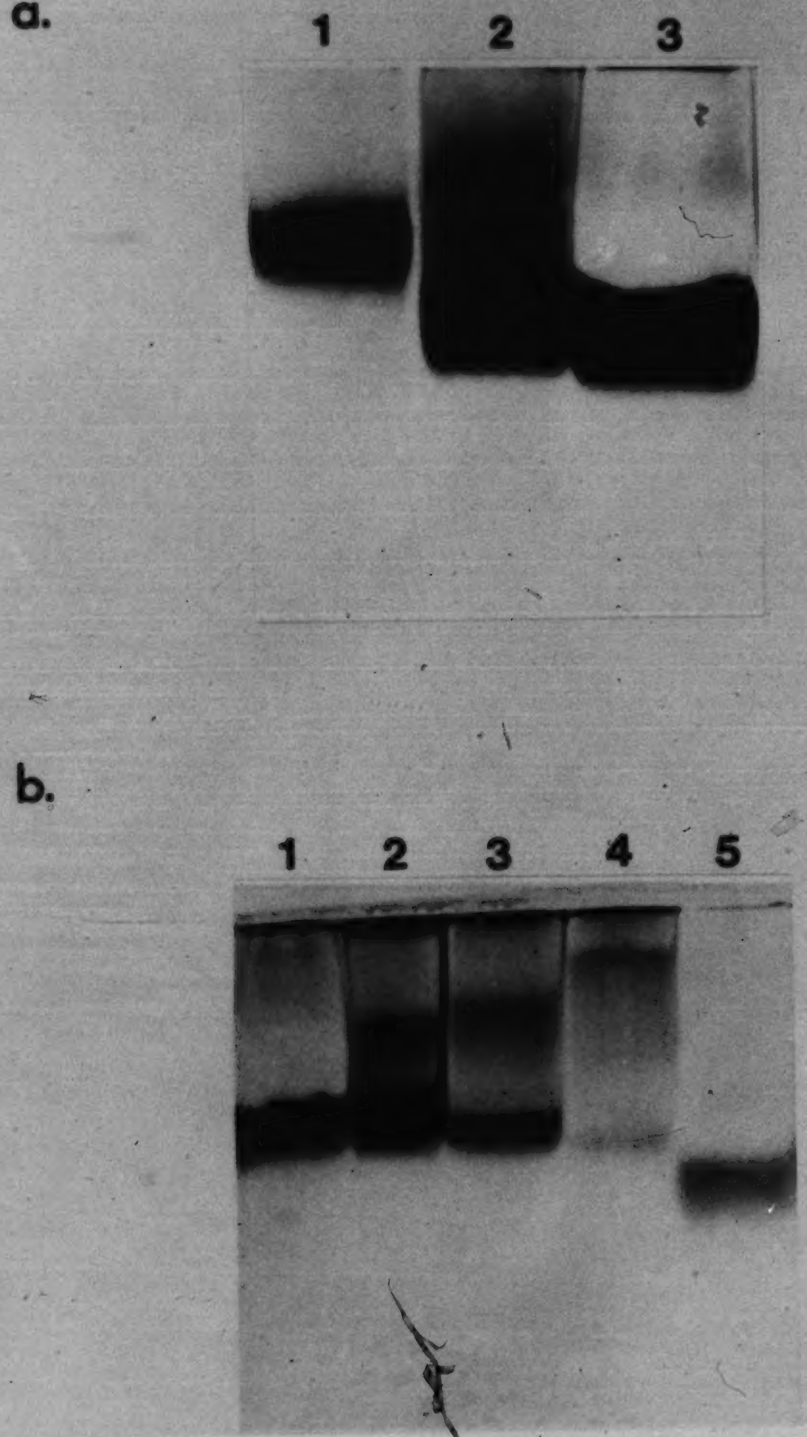

a.
1
2
3
b.
1
2
3
4
5

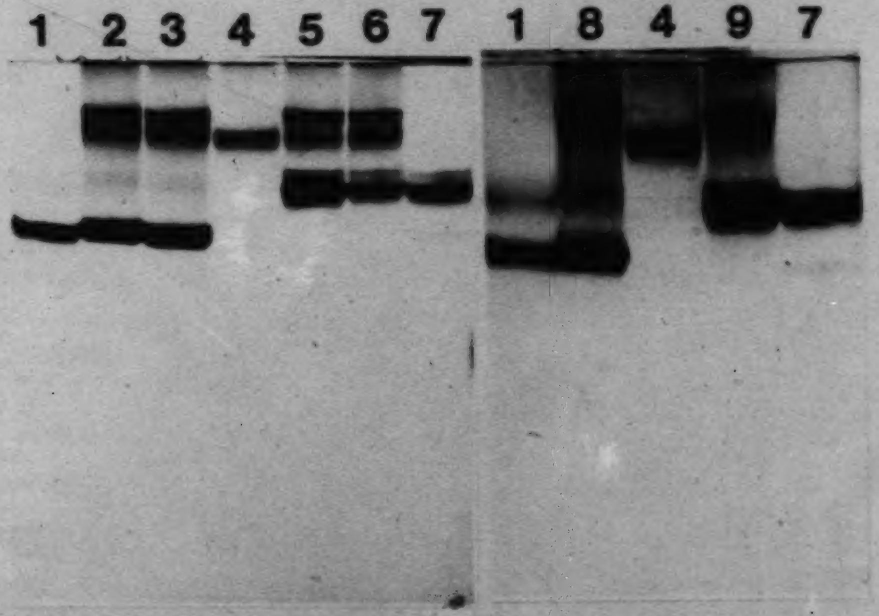
Fig. 34a. Interactions of T1 and T2 with TnCT

(1) T2
(2) T2 + TnCT (0.75:1)
(3) T2 + TnCT (1.5:1)
(4) TnCT
(5) T1 + TnCT (1.5:1)
(6) T1 + TnCT (0.75:1)
(7) T1
(8) T2 + TnCT (3:1)
(9) T1 + TnCT (3:1)

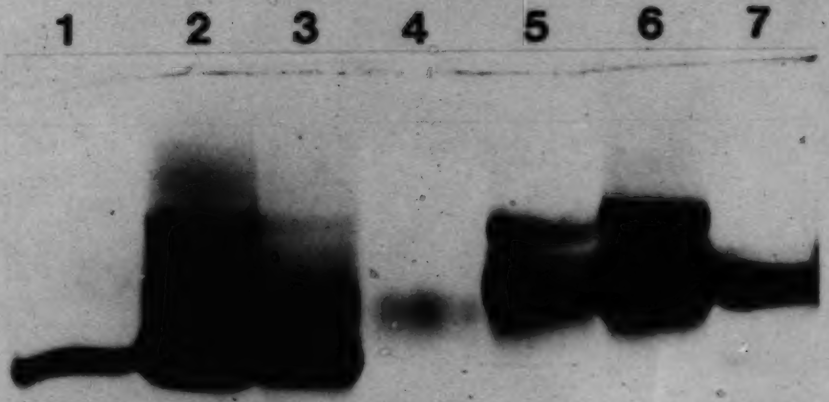
Fig. 34b. Interactions of T1 and T2 with CB1

(1) T2
(2) T2 + CB1 (2.5:1)
(3) T2 + CB1 (1:1)
(4) CB1
(5) T1 + CB1 (1:1)
(6) T1 + CB1 (2.5:1)
(7) T1

a.


1
2
3
4
5
6
7
1
8
4
9
7

b.



on an SDS-urea gel together with T1, T2 and CB1 bands treated in the same manner. Fig. 35a clearly demonstrates that the complex bands obtained from mixtures of T1 or T2 and CB1 were composed of their respective components.

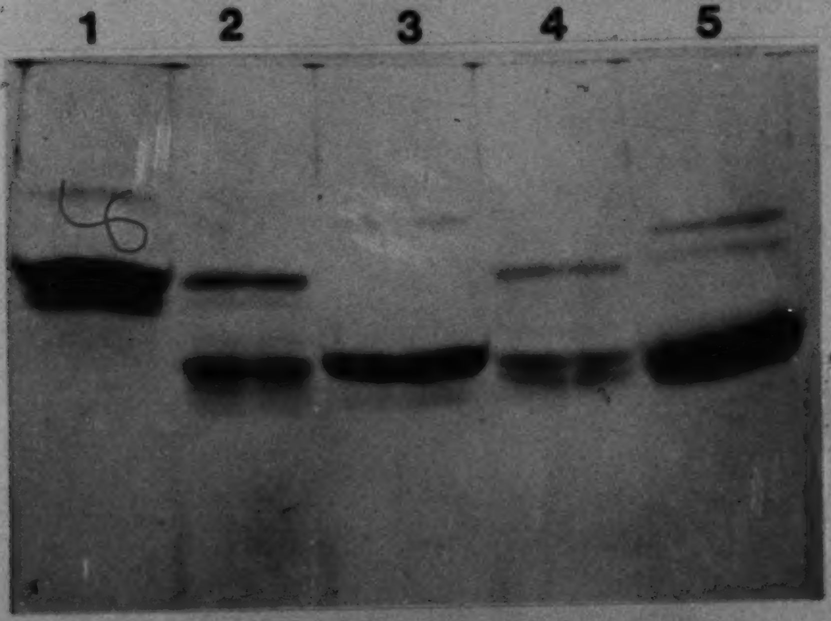
An objection arising from this technique was the probability of diffusion of certain proteins from the cut-off gel during the treatment process. Thus the solutions in which the gels were soaked were also applied to a SDS-urea polyacrylamide gel. The electrophoretic patterns (Fig. 35b) observed from this experiment were identical to those obtained when the treated gel was used in the second dimensional run indicating non-selective diffusion of proteins from the gel during the treatment process. This technique has been found to be more convenient and as good, if not better, than the usual procedure of eluting the protein from a mashed gel (Weber & Osborn, 1975).

In a similar manner, the binding properties of the other fragments T3, T4, CN1A, CN1B, CT1 and Cy1 with both TnCT and CB1 were determined (Fig. 36-39) and the results summarized in Table VI. None of these fragments exhibited binding to either TnCT or CB1. This observation was surprising because T3 and CN1A are only a few amino acids shorter than T1 as is CN1B compared with T2. The fact that both Cy1 and CT1, which contain the whole sequence of T1, showed no binding with either TnCT or CB1 led us to consider this technique non-specific and unreliable. Thus another technique for the interaction studies was explored.

Fig. 35. Analysis of the composition of the complex between T1 or T2 and CB1 on second dimensional polyacrylamide gel. The bands of the fragments and their complexes cut out from the benign gel were soaked in 1% SDS, 6M urea, 1% mercaptoethanol, 50 mM sodium phosphate, pH 7.0 for 36 hours at 34°C. The treated bands (a) and the solution they were soaked in (b) were rerun in SDS-urea polyacrylamide gels.

(1) T2
(2) T2 + CB1 complex
(3) CB1
(4) T1 + CB1 complex
(5) T1

a.



b.

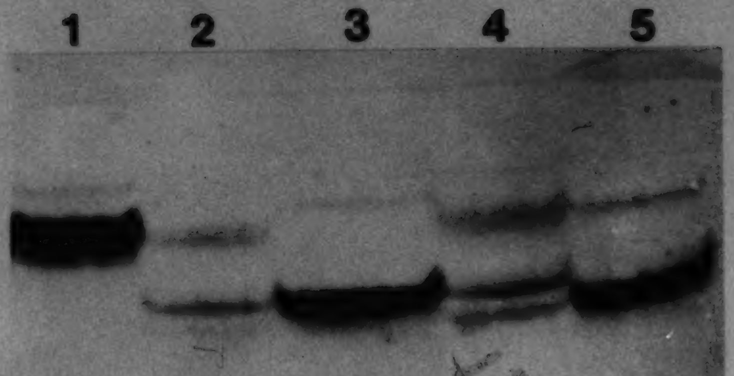


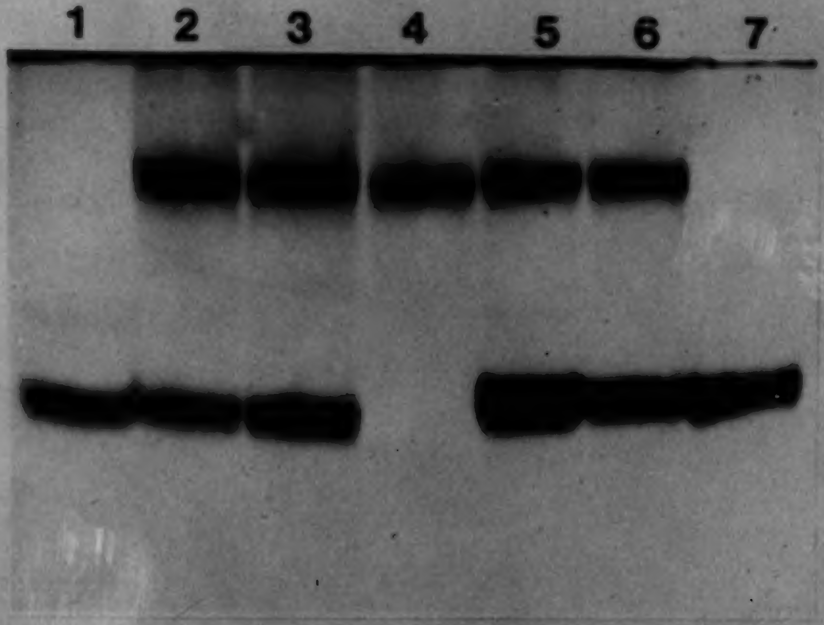
Fig. 36a. Interactions of T3 and T4 with TnCT

(1) T4
(2) T4 + TnCT (1.5:1)
(3) T4 + TnCT (3:1)
(4) TnCT
(5) T3 + TnCT (3:1)
(6) T3 + TnCT (1.5:1)
(7) T3

Fig. 36b. Interactions of T3 and T4 with CB1

(1) T3
(2) T3 + CB1 (2:1)
(3) T3 + CB1 (1:1)
(4) T3 + CB1 (0.5:1)
(5) CB1
(6) T4 + CB1 (0.5:1)
(7) T4 + CB1 (1:1)
(8) T4 + CB1 (2:1)
(9) T4

a.



b.

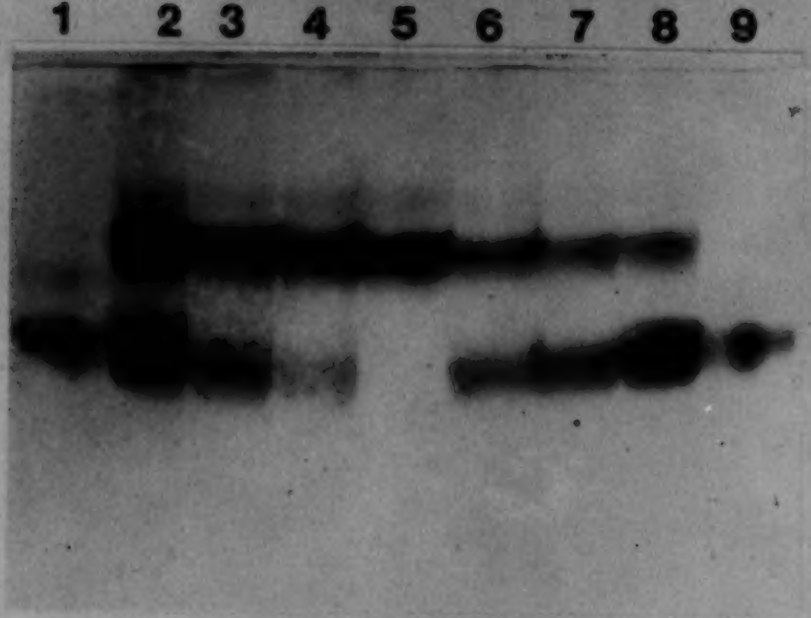
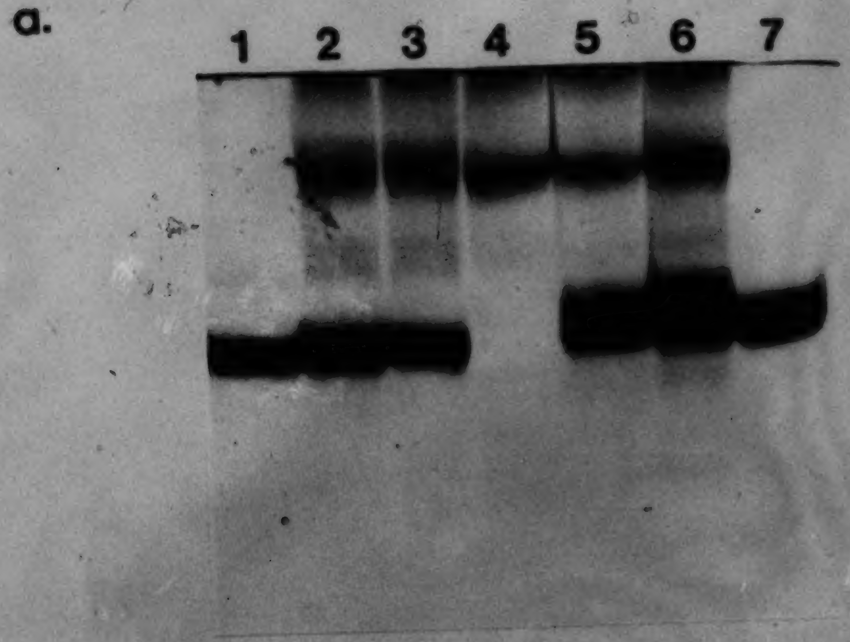


Fig. 37a. Interactions of CN1A and CN1B with TnCT

(1) CN1A
(2) CN1A + TnCT (3:1)
(3) CN1A + TnCT (1.5:1)
(4) TnCT
(5) CN1B + TnCT (1.5:1)
(6) CN1B + TnCT (3:1)
(7) CN1B

Fig. 37b. Interactions of CN1A and CN1B with CB1

(1) CN1A
(2) CN1A + CB1 (5:1)
(3) CN1A + CB1 (2.5:1)
(4) CN1A + CB1 (0.5:1)
(5) CB1
(6) CN1B + CB1 (0.5:1)
(7) CN1B + CB1 (2.5:1)
(8) CN1B + CB1 (5:1)
(9) CN1B


a.
1
2
3
4
5
6
7

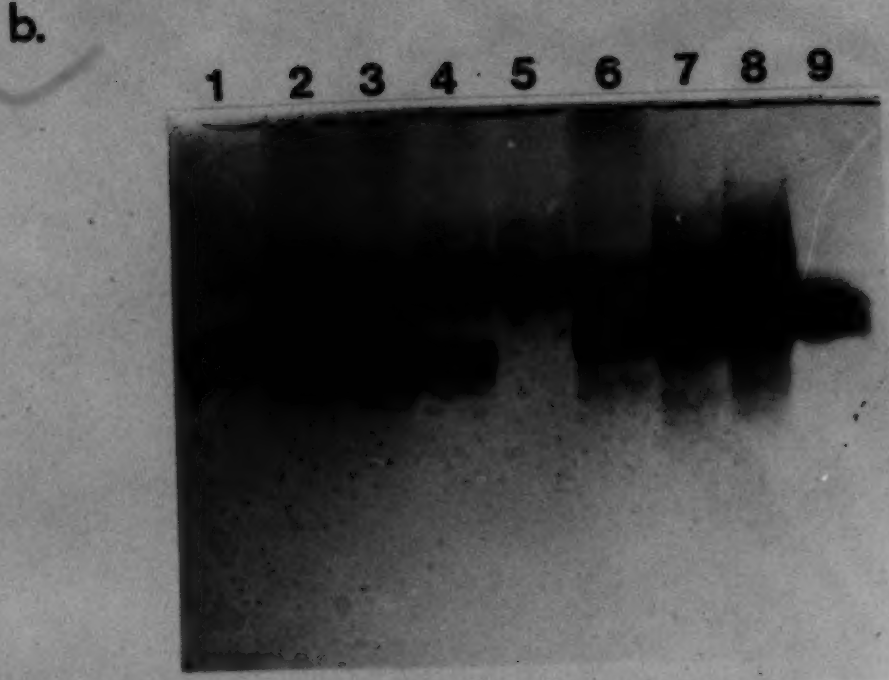

b.
1
2
3
4
5
6
7
8
9

Fig. 38. Interactions of CT1 and CT2 with CB1

(1) CT2
(2) CT2 + CB1 (1.4:1)
(3) CB1
(4) CT1 + CB1 (1:1)
(5) CT1 + CB1 (2:1)
(6) CT1

Fig. 39. Interactions of Cy1 and Cy2 with CB1

(1) Cy1
(2) Cy1 + CB1 (0.5:1)
(3) Cy1 + CB1 (1:1)
(4) Cy1 + CB1 (2:1)
(5) CB1
(6) Cy2 + CB1 (0.5:1)
(7) Cy2 + CB1 (1:1)
(8) Cy2 + CB1 (2:1)
(9) Cy2

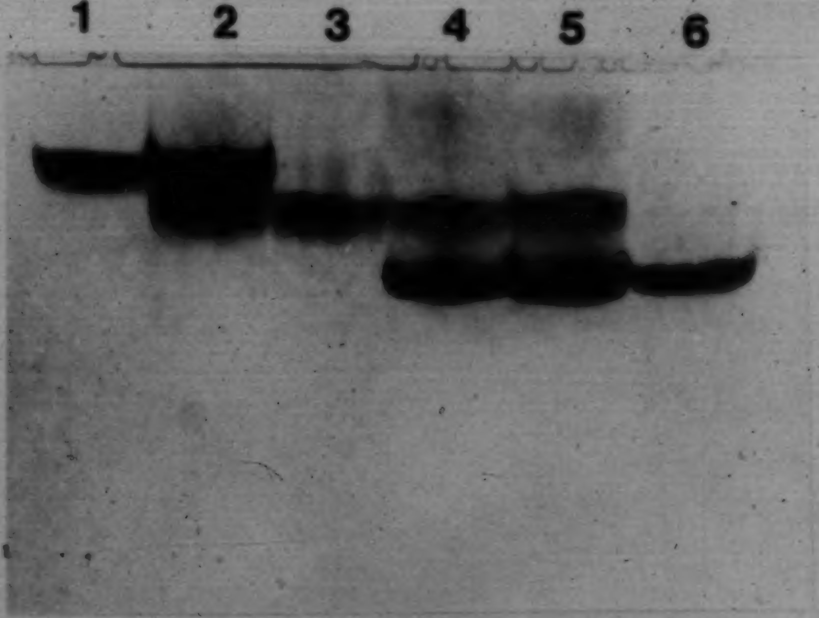


Fig. 38

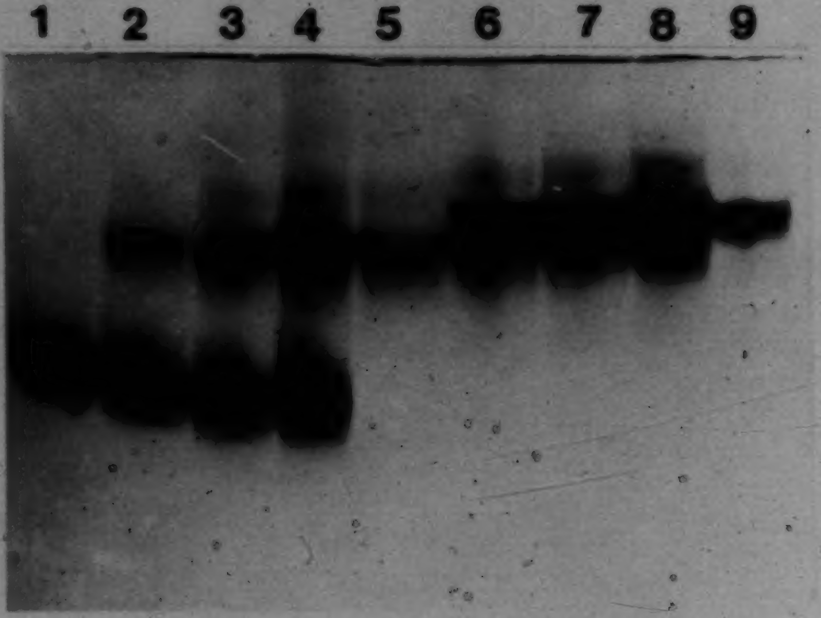


Fig. 39

TABLE VI

Binding of αTm and its Fragments to TnCT and CB1 Determined by Polyacrylamide Gel Electrophoresis

	Residue Numbers	Complex Formation	
		TnCT	CB1
αTm	1-284	+	+
NH_2-terminal fragments			
T1	1-133	+	+
T3	13-125	-	-
CN1A	11-127	-	-
CT1	1-169	-	-
Cyl	1-189	-	-
COOH-terminal fragments			
T2	134-284	+	+
T4	183-284	-	-
CN1B	142-281	-	-

(c) Gel Filtration

The formation of a complex between two proteins may be detected by molecular sieving since the complex will have a greater molecular size than its components and will be eluted earlier than the individual proteins.

A column of Biogel A 0.5M was used in this study. The samples were dissolved and eluted with 0.15M KCl, 1 mM DTT, 0.01 mM $CaCl_2$ 10 mM sodium phosphate buffer, pH 7.0. A mixture of CB1 and αTm resolved into a peak eluted earlier than αTm and CB1, and another peak which coincided with the elution peak of αTm alone (Fig. 40). Analysis of the peaks composition on SDS-urea polyacrylamide gel revealed that the early peak was composed of CB1 and αTm while the latter peak was pure αTm.

Since this technique proved to be feasible, the binding properties of the αTm fragments, T1, T2, CN1A, CN1B, CT1 and CT2 to CB1 were determined. The elution profile of T1 and CB1 showed a peak in front of the T1 peak (Fig. 41) which was shown by SDS-urea polyacrylamide gel to contain CB1 and T1. When a mixture of T2 and CB1 was passed through the column, the CB1 peak was not detected but instead a peak of T2 as well as a shoulder in front of it was observed (Fig. 42). Aliquots of fractions across the peak were run in SDS-urea polyacrylamide gel and this indicated the presence of CB1 and T2 in the shoulder of the peak. Elution profiles of mixtures of CB1 with Cy1, CT1, CN1A and CN1B (Figs. 43-46) showed no complex

7

Figs. 40-46.

Gel filtration of CB1, αTm and its fragments and their mixtures on Biogel A 0.5m. The sample, dissolved in 0.15M KCl, 1 mM DTT, 0.01 mM $CaCl_2$, 10 mM sodium phosphate buffer, pH 7.0, was applied to the column (0.9 x 105 cm) equilibrated at 3.5 ml/hr operated at room temperature. Elution was done with the same buffer and monitored by the absorbance at 230 nm of the 1 ml fractions.

Fig. 40. Interaction of αTm with CB1

a. CB1 (0.5 mg)
b. αTm (0.5 mg)
c. CB1 (0.5 mg) + αTm (3.7 mg)

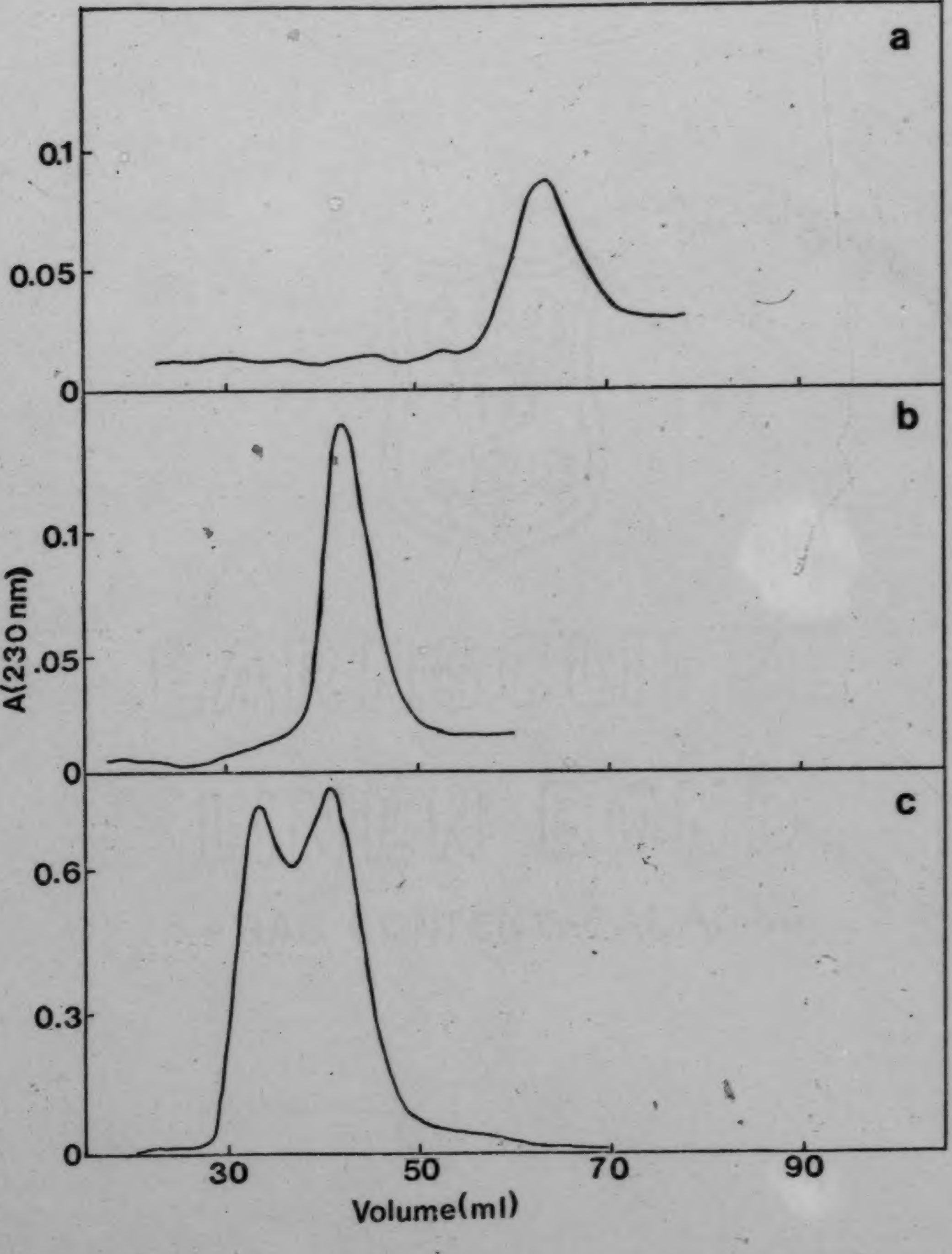

a
0.1
0.05
0
b
A(230 nm)
0.1
.05
0
c
0.6
0.3
0
30
50
70
90
Volume(ml)

Fig. 41. Interaction of T1 with CB1

a. CB1 (0.5 mg)
b. T1 (0.5 mg)
c. CB1 (0.5 mg) + T1 (2.1 mg)

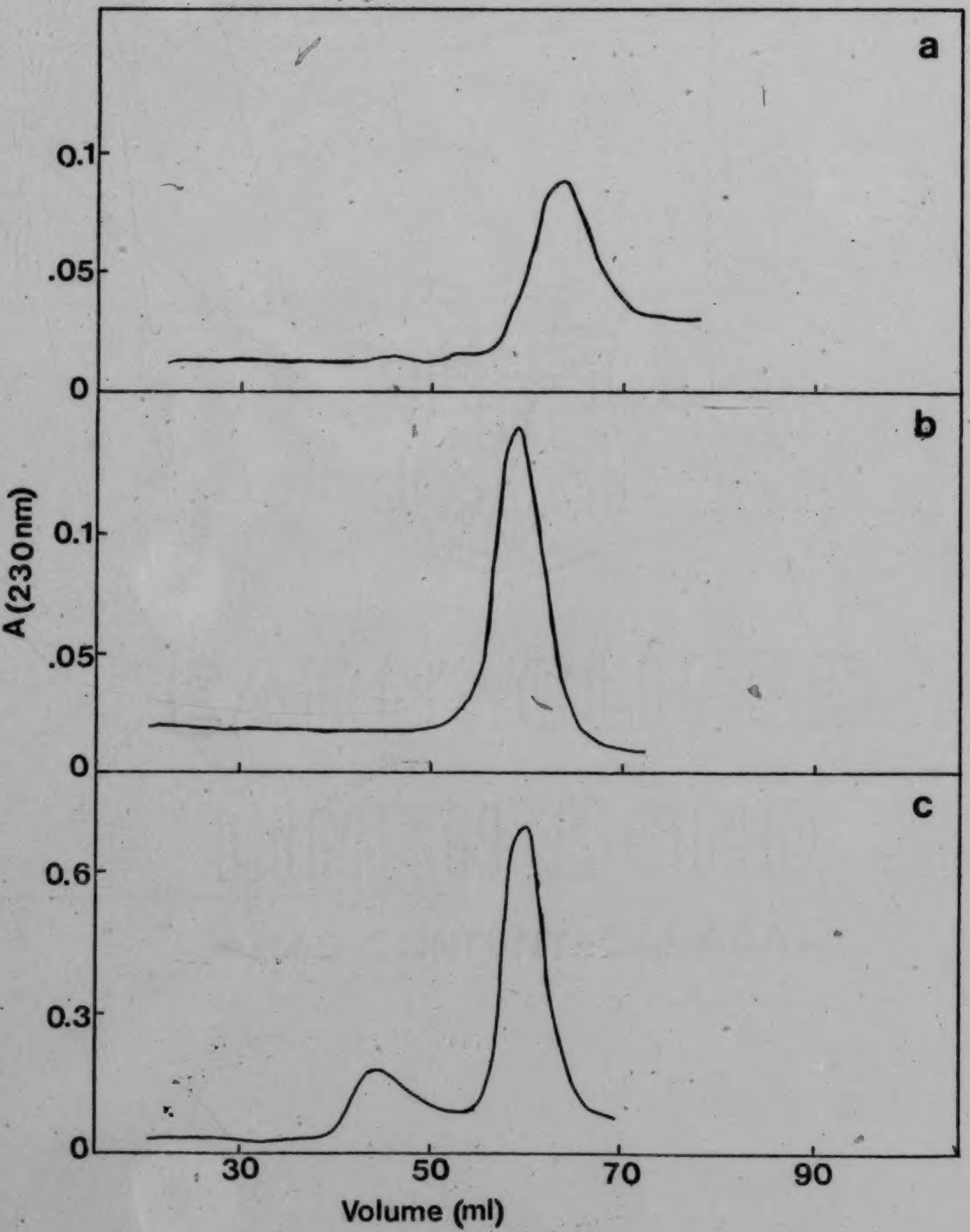

a
b
c
A (230nm)
0.1
.05
0
0.1
.05
0
0.6
0.3
0
30
50
70
90
Volume (ml)

Fig. 42. Interaction of T2 with CB1

a. CB1 (0.5 mg)
b. T2 (0.5 mg)
c. CB1 (0.5 mg) + T2 (2.4 mg)

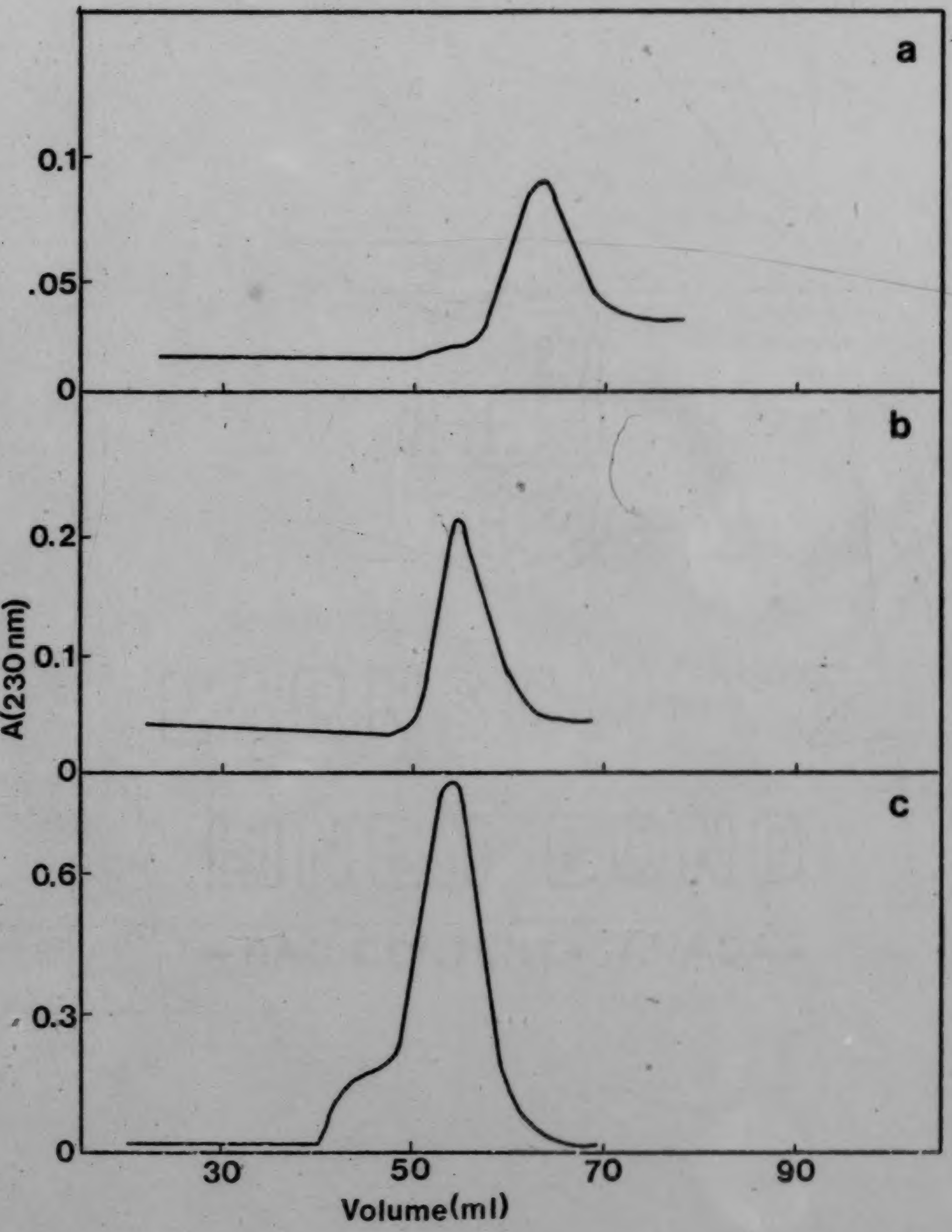

a
0.1
.05
0
b
0.2
0.1
0
A(230 nm)
c
0.6
0.3
0
30
50
70
90
Volume(ml)

Fig. 43. Interaction of CN1A with CB1

a. CB1 (0.5 mg)
b. CN1A (0.7 mg)
c. CB1 (0.5 mg) + CN1A (1.85 mg)

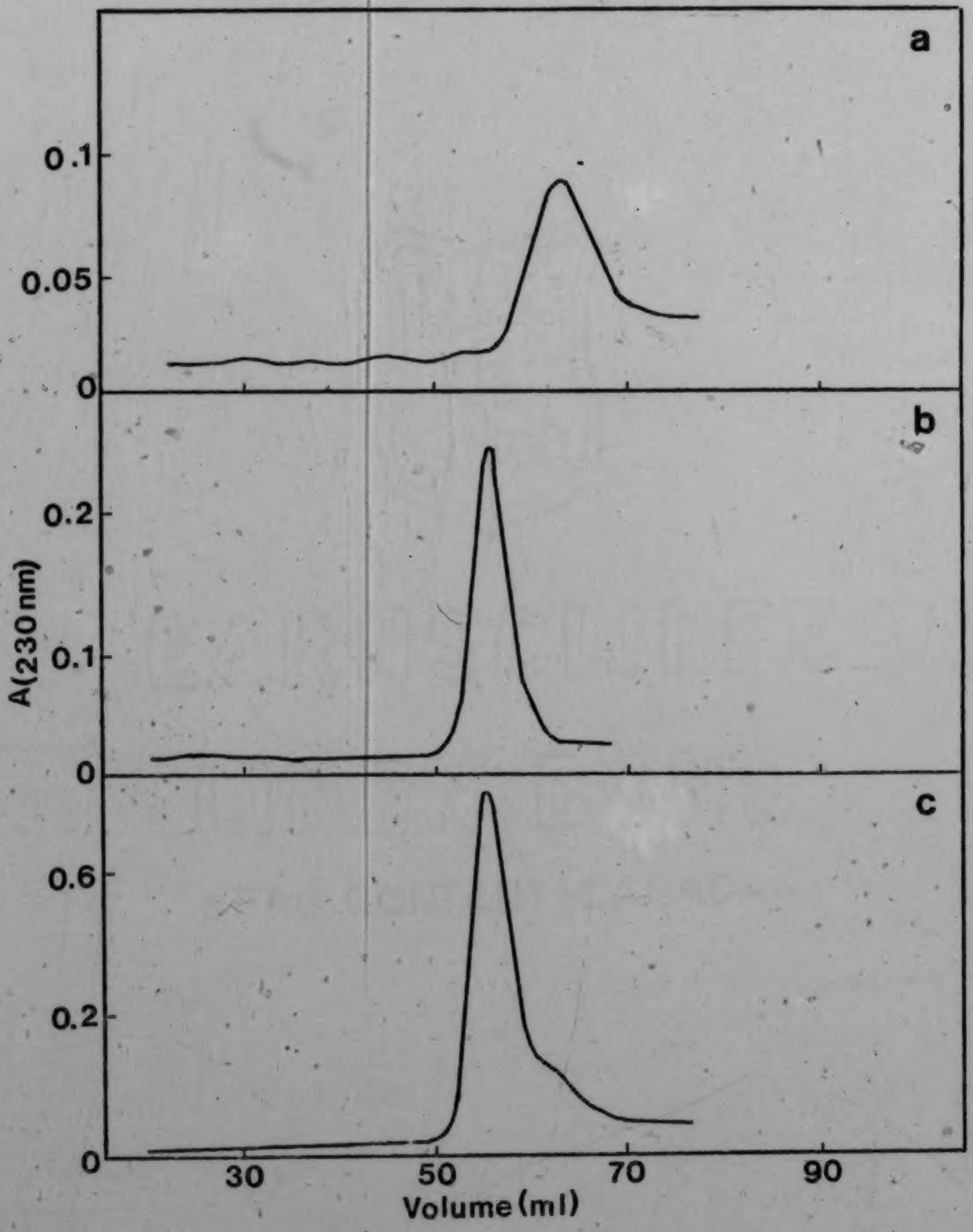

a
0.1
0.05
0
b
0.2
0.1
0
c
0.6
0.2
0
A(230 nm)
30
50
70
90
Volume (ml)

Fig. 44. Interaction of CN1B with CB1

a. CB1 (0.5 mg)
b. CN1B (0.8 mg)
c. CB1 (0.5 mg) + CN1B (2.3 mg)

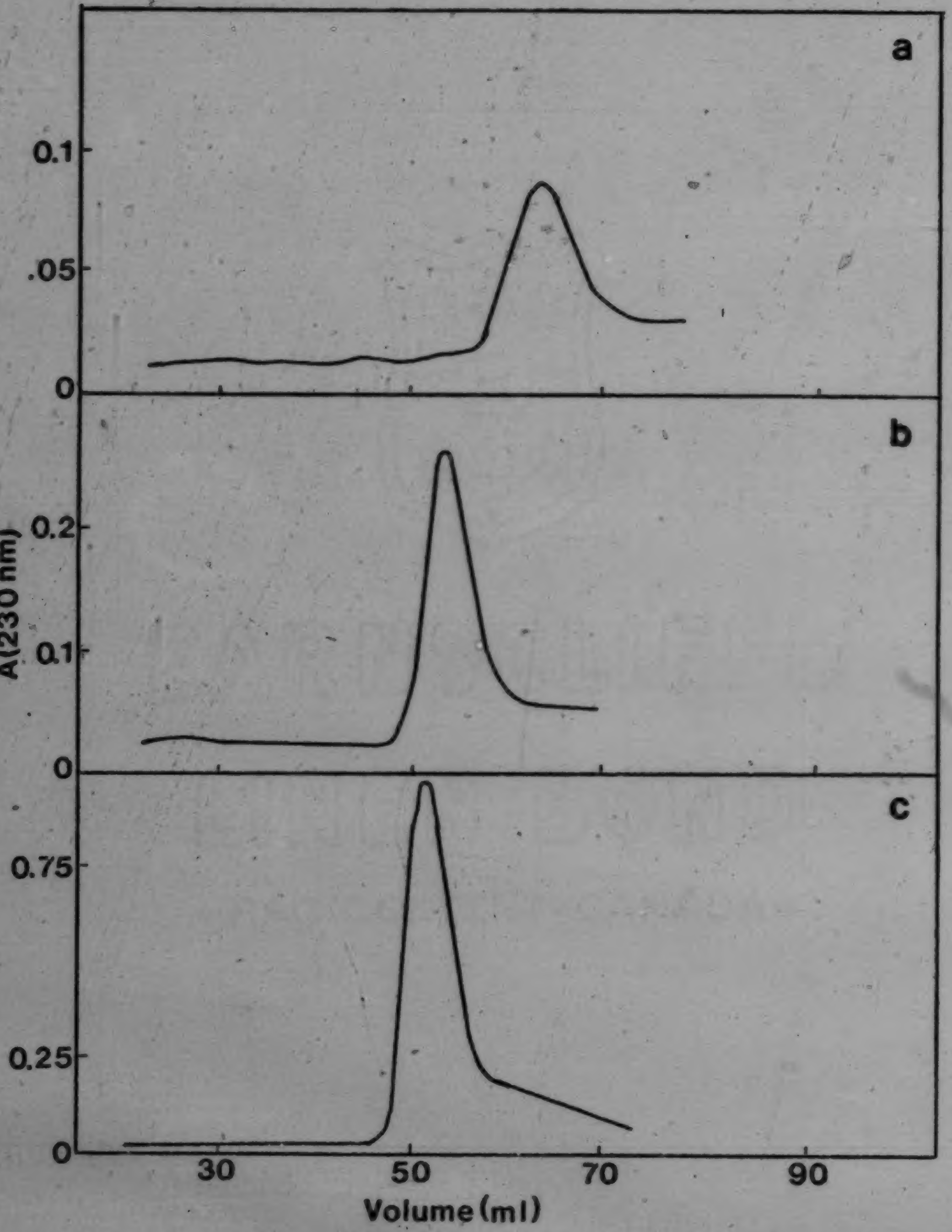

a
0.1
.05
0
b
0.2
0.1
0
c
0.75
0.25
0
A(230 nm)
30
50
70
90
Volume (ml)

Fig. 45. Interaction of CT1 with CB1

- a. CB1 (0.5 mg)
- b. CT1 (0.5 mg)
- c. CB1 (0.5 mg) + CT1 (2.1 mg)

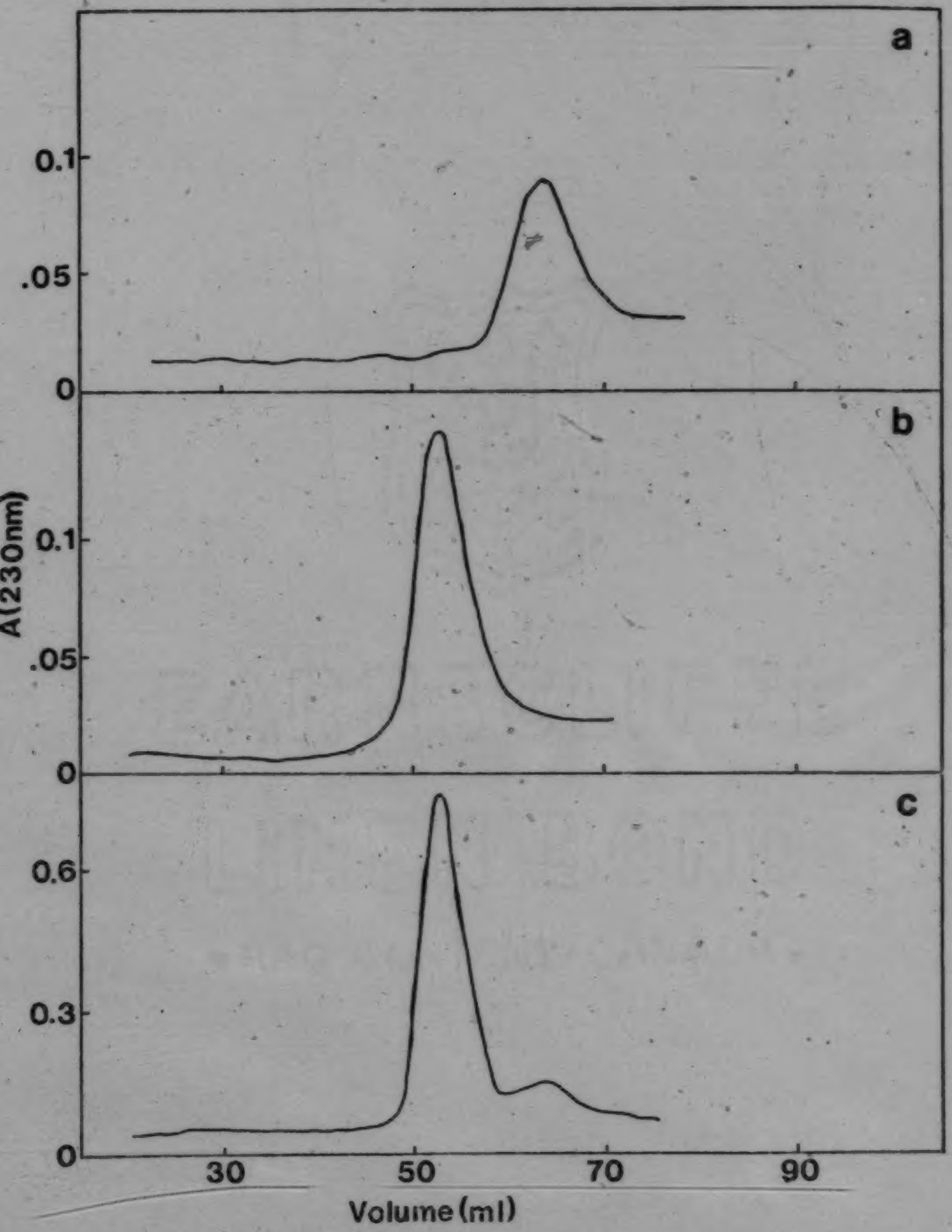
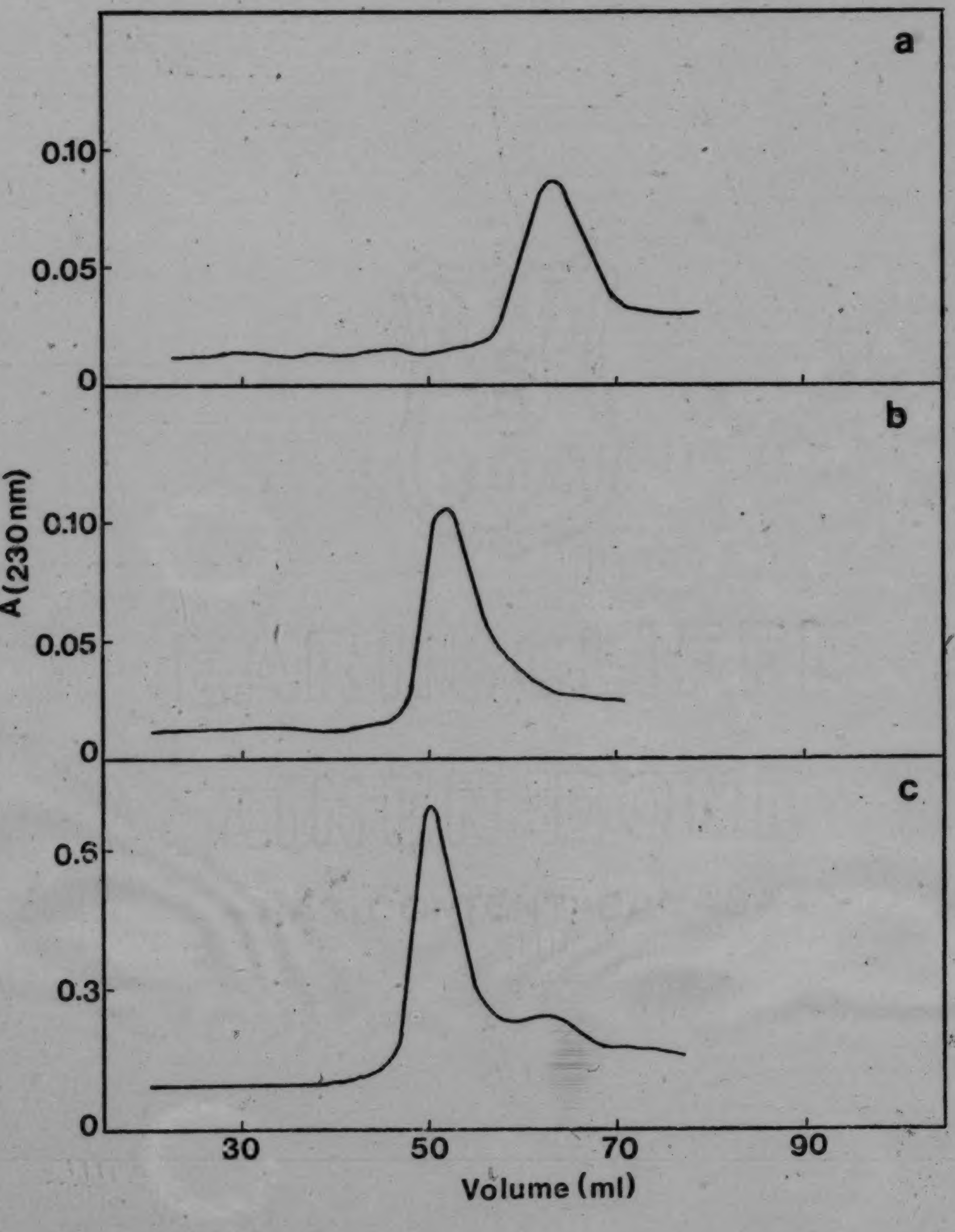

a
b
c
A(230nm)
0.1
.05
0
0.1
.05
0
0.6
0.3
0
30
50
70
90
Volume (ml)

Fig. 46. Interaction of Cyl with CB1

a. CB1 (0.5 mg)
b. Cyl (0.5 mg)
c. CB1 (0.5 mg) + Cyl (2.4 mg)


a
b
c
0.10
0.05
0
0.10
0.05
0
0.6
0.3
0
A(230 nm)
30
50
70
90
Volume (ml)

formation between the components of the mixtures as shown by the elution profiles and SDS-urea polyacrylamide gel electrophoresis of aliquots of fractions across the peak.

The results obtained using the technique (Table VII) were exactly the same as obtained from polyacrylamide gel electrophoresis experiments and again, the same inconsistency occurred. The reasons for these observations were not clear at this time.

(d) Affinity Chromatography

Binding studies between two proteins by affinity chromatography require the covalent coupling of one of the proteins to a solid matrix which is then packed into a column. If the other protein sticks to the column when passed through it indicates interaction between the proteins. The nature of this interaction can be deduced from the method of elution required. Agarose resin, Sepharose 4B with or without spacer groups, was employed as the solid matrix for the affinity columns. The resin with spacer group was chosen to provide flexibility for interaction of the ligands with other proteins.

1. Aminohexyl Sepharose

Control experiments were performed to test the inertness of the resin towards some proteins used in the study. Aminohexyl (AH) Sepharose 4B was swollen, packed in a column and tested with αTm. αTm was observed to bind

TABLE VII

Binding of αTm and its Fragments to CB1 Determined by Gel Filtration

	Residue Numbers	Complex Formation
αTm	1-284	+
NH_2-terminal fragments		
T1	1-133	+
T3	13-125	-
CN1A	11-127	-
CT1	1-169	-
Cy1	1-189	-
COOH-terminal fragments		
T2	134-284	+
T4	183-284	-
CN1B	142-281	-

to the column and was eluted only after the ionic strength of the eluent was increased. Under the conditions of the experiment, the amino groups in the resin are ionized and may have interacted electrostatically with the acidic αTm. This explanation was invalidated when some acidic proteins, BSA, ovalbumin and myoglobin, as well as the basic protein cytochrome C (Table VIII), passed through the column without being retarded. Since electrostatic attraction had been ruled out by these experiments, the other obvious difference between αTm and BSA is in their conformation. A variety of helical polypeptides were applied to the column to verify whether the resin is specific for helical proteins. All of the proteins tested bound to the column to different extents (Table VIII). These experiments appeared to demonstrate some correlation between the helical contents of the proteins tested and their binding to AH-Sepharose. The only apparent exception noted to this observation was for Tn. The Tn complex is globular but does contain a high proportion of helical content in certain localized regions of the structure of its three components, particularly in the case of TnT (Pearlstone & Smillie, 1977).

Because of these properties, AH-Sepharose was judged unsatisfactory as a matrix for examining the binding of αTm and its fragments to Tn. These results demonstrate the necessity of carefully examining the binding properties of the solid support media used in affinity chromatography and raise questions as to the validity of some results reported

TABLE VIII

Binding of Various Proteins to Aminohexyl-Sepharose 4B

	Elution [KCl], M
Cardiac Tm	0.35
Platelet Tm	0.35
CN1A	0.25
CN1B	0.29
LMM CNBr fragments	0.35
Troponin	0.22
Ovalbumin	0
Bovine serum albumin	0
Cytochrome C	0

in the literature where appropriate controls have not been performed. For example in a recent publication, Higashi and Hirabayashi (1978), described the interaction studies between shellfish adductor muscle Tm coupled to AH-Sepharose and rabbit skeletal Tn. Partly on the basis of the observation that Tn bound to the column, they made a conclusion that the two proteins interacted.

2. Carboxyhexyl-Sepharose

Unlike AH-Sepharose 4B, carboxyhexyl (CH)-Sepharose 4B, did not bind αTm or BSA. Tn was coupled to the carboxyhexyl groups and their binding to αTm and its fragments were tested at room temperature. It was observed that αTm and the fragments bound to the column while BSA and ovalbumin were eluted at the void volume. αTm was eluted at 0.2M KCl whereas its fragments were eluted at lower ionic strength. The stability of the ligands and reproducibility of the results were determined by running αTm through the column periodically. These experiments revealed that binding of αTm to the ligand became weaker with time. This may be due to degradation of the ligand or irreversible binding of some proteins to the column and its gradual "poisoning". To distinguish between these two possibilities, CH-Sepharose-Tn was prepared and packed into two columns. The binding property of one column to αTm was determined and both columns were stored at 4°C for a week, after which their binding capacities to αTm were checked again. The ionic strength required to elute αTm from both columns was the same but lower than the observed value from the freshly made column. These experiments indicated that Tn underwent degradation with time and storing the columns at 4°C did not prevent the process. In view of these results, CH-Sepharose was not used for the interaction studies.

3. CNBr Activated Sepharose

Since no success was obtained with Sepharose 4B with spacer groups, the possible use of CNBr activated Sepharose 4B without spacer groups was explored. To test whether the matrix was inert towards αTm, it was swollen in 1 mM HCl overnight at 4°C and deactivated by shaking with 1M ethanolamine for 1 1/2 hours at room temperature. αTm did not bind to the deactivated matrix so Tn, TnT and CB1 were covalently bound to the support. A rough estimate of the amount of ligands bound to the Sepharose showed 75% coupling.

The interaction of αTm with the immobilized Tn, TnT and CB1 were determined in the same manner as with the previous columns; that is, the column was washed with at least 15 ml of 10 mM imidazole, 1 mM DTT, 1 mM EGTA, pH 7.0 solution after application of sample and then a salt gradient of 0-0.5M KCl was established. The salt concentration of the fraction at the peak of the eluted protein was taken as a measure of the strength of binding of the protein to the ligand. In the course of the study, five different preparations of TnT-Sepharose were used and all preparations showed the same results with regards to their binding properties to αTm and its fragments. A periodical check of the strength of binding of αTm to the columns did not show detectable signs of degradation.

αTm bound to the column and was eluted only when the salt concentration was raised to 0.20M. The presence of Mg^{++} or Ca^{++} in the eluent did not affect the interaction between αTm and TnT confirming previous findings (Potter & Gergely, 1974, and Kawasaki & van Eerd, 1972). Similarly, omission of DTT from the solvent did not affect the binding indicating that disulfide formation is not involved in the interaction of the two proteins.

These experiments showed that at higher ionic strength than 0.20, the interaction between αTm and TnT, Tn or CB1 ceases, which is contradictory to other studies (Jackson, et al., 1975) where binding could still be detected at higher salt concentrations. The weaker interaction with the immobilized proteins may be due to steric factors or may be due to coupling to the resin of some amino groups on the protein which are involved in binding to αTm.

The binding capacity of a TnT-Sepharose column (3 ml) was determined by applying known amounts of αTm to the column. The amount of αTm applied when a slight excess of the protein was eluted at the void volume was taken as the maximum binding capacity. The observed value, 0.8 mg, was very low considering that a rough estimate of 6 mg of TnT was covalently bound to the resin. Coupling of the amino groups of the ligand to Sepharose is a random process and the observed results suggest that it has significantly blocked some of the binding sites and/or caused steric inhibition of the interaction of αTm with Tn, TnT and CB1.

The binding properties of various proteins as well as fragments of αTm to TnT-Sepharose were determined and are listed in Table IX. Representative elution profiles of samples applied to the TnT-Sepharose column are illustrated in Fig. 47. BSA was not retarded by the column and was eluted at the void volume while CN1A, CN1B and αTm were eluted only after a salt gradient was established at 0.05, 0.10 and 0.20M KCl, respectively. Other globular acidic proteins tested, ovalbumin and myoglobin, passed through the column without being retarded whereas cytochrome C, a basic globular protein, helical CNBr fragments of light meromyosin (LMM), and fragments of αTm bound to the column. These samples were eluted from the column at different ionic strengths indicating different extents of interaction with the ligand.

The binding properties of αTm and some of its fragments to immobilized Tn, TnT and CB1 were observed to be similar suggesting that the nature of binding between αTm and Tn was not affected by conformational changes resulting from dissociation of the Tn complex into subunits nor by fragmentation of TnT.

On the basis of their binding properties, the αTm

TABLE IX

Binding of αTm, its Fragments and Other Proteins to Immobilized Tn, TnT and CB1

	Residue Numbers	Elution [KCl] M		
		TnT-Sepharose	CB1-Sepharose	Tn-Sepharose
Bovine serum albumin		0	0	
Ovalbumin		0	0	
Myoglobin		0		
Cytochrome C		0.04		
LMM CNBr fragments		0.06		0.07
αTm	1-284	0.20	0.20	0.20
NH_2-terminal fragments				
T1	1-133	0.05	0.04	0.07
T3	13-125	0.05	0.03	
CN1A	11-127	0.05	0.02	0.05
CT1	1-169	0.05	0.04	
Cy1	1-189	0.05	0.03	
COOH-terminal fragments				
T2	134-284	0.10	0.11	
T4	183-284	0.10		
T5	183-244	0		
CN1B	142-281	0.10	0.10	
CT2	170-284	0.10		0.12
Cy2	190-284	0.10	0.10	
Non-polymerizable Tm	1-276	0.10		

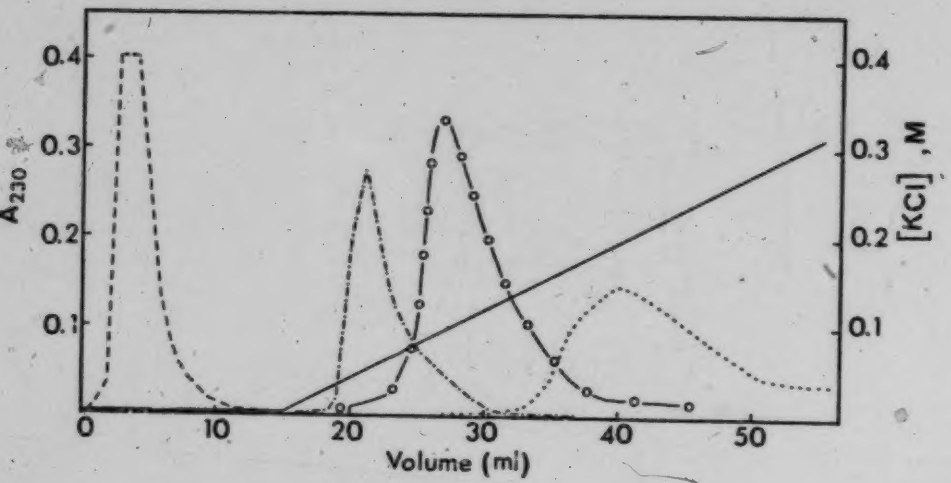


Fig. 47. Elution profiles of bovine serum albumin (---) CN1A (-·-·-), CN1B (-o-o-) and αTm (·····) on a TnT-Sepharose affinity column (0.9 x 8 cm). After application of the samples, the column was eluted with at least 2 column volumes of starting buffer before establishing a gradient of 0 - 0.5M KCl (-).

fragments can be divided into two distinct classes: fragments derived from the NH_2-terminal half of the molecule which dissociates from the ligand at about 0.05M KCl and the COOH-terminal fragments which bind more strongly to the ligand and are eluted at about 0.1M salt with the exception of T5.

The fact that the fragments can be eluted from the column by increasing the ionic strength of the eluent means that the interaction between the proteins is mainly due to electrostatic attraction. But the binding of the NH_2-terminal fragments to the ligand cannot be attributed to purely electrostatic factors because other acidic proteins, BSA, ovalbumin and myoglobin did not bind to the column. The observation that cytochrome C bound to the column points out that, if anything, the immobilized TnT binds basic proteins. The contribution of the conformation of the sample to the strength of binding is reflected by the observation that CNBr fragments of LMM bound to the column to the same extent as the NH_2 terminal fragments. This suggests that the interaction of the NH_2-terminal fragments to the ligand is non-specific. On the other hand, the fragments derived from the COOH-terminal half of the molecule exhibited higher affinity for the ligand in spite of the fact that most of them are shorter and all of them are less helical than the NH_2-terminal fragments. The difference in the binding affinity of the COOH-terminal fragments is taken to be due to a more specific interaction of these fragments and the ligands. The data obtained indicate that the binding region is between residues 190 and 276, which is consistent with the hypothesis that the Tn binding site is one-third from the C-terminus of the molecule.

Unfortunately, T5 had low α-helical structure so that its lack of binding to immobilized TnT could not be interpreted in terms of its lack of a binding site for Tn. Otherwise the binding region could possibly have been delineated more precisely.

Non-polymerizable Tm is only a few amino acids shorter than αTm so it was expected to bind to immobilized TnT as tightly as the intact molecule but it exhibited the same affinity as the COOH-terminal fragments. This could be accounted for by the ability of αTm to polymerize into long filaments with multiple binding sites, a property which non-polymerizable Tm has lost. Based on this experimental evidence it is not necessary to invoke a more direct role for the C-terminus of Tm in the binding of Tn.

However, the possible importance of an indirect role of the head to tail overlap of Tm molecules in the binding of Tn is indicated by the remarkable viscosity increases that occur when Tn, TnT and certain TnT fragments are complexed with Tm in solution and which are abolished with non-polymerizable Tm. If one accepts that the binding site for TnT or Tm is centered about one-third of the distance from the C-terminus, then this phenomenon can only be understood in terms of a conformational change induced by the binding of Tn and transmitted to the head to tail overlap along the length of the coiled coil structure. If such changes in the region of the putative Tn binding site can affect the extent of head to tail aggregation of Tm molecules, then the reverse phenomenon, namely, an effect of the degree of polymerization on the binding affinity of Tm for Tn can be anticipated, an effect which is consistent with the results of this study (Pato & Smillie, 1978).

To evaluate this hypothesis, various combinations of the fragments were mixed and applied to the TnT-Sepharose column. In all cases where fragments from the NH_2- and COOH-terminal half of the molecule were combined, two peaks emerged from the column (Figs. 48 and 49). The first peak was eluted at approximately 0.05M KCl, the salt concentration required to dissociate the NH_2-terminal fragments from the ligands while the second peak was eluted at concentrations ranging from 0.10 to 0.15M KCl (Table Xa). The material from the peaks were dialyzed against water, freeze dried and examined in SDS-urea polyacrylamide gels. Care was taken in pooling the fractions so that the material from the peaks were not cross-contaminated. It was observed that the first peak was always composed of the NH_2-terminal fragments whereas the second peak was composed of just the COOH-terminal fragments in some cases (Fig. 48b) and both the NH_2- and COOH-terminal fragments in others (Fig. 49b).

Fig. 48a. Elution profile of mixture of T3 and CN1B (0.5 mg each) on a TnT-Sepharose affinity chromatography column. Same experimental conditions were used as in Figure 47. Fractions were pooled as indicated.

Fig. 48b. SDS-urea polyacrylamide gel of the fractions I and II.

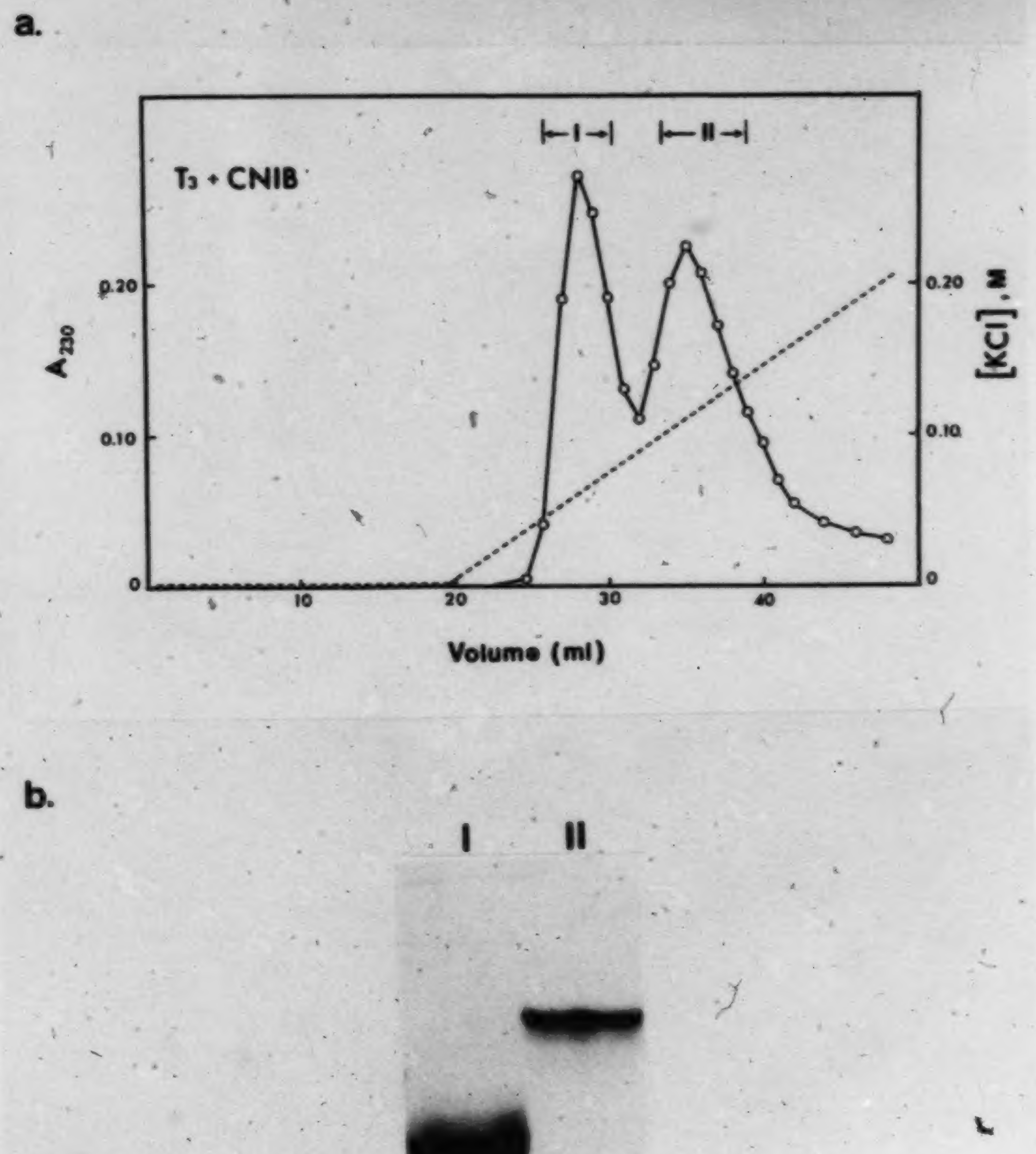
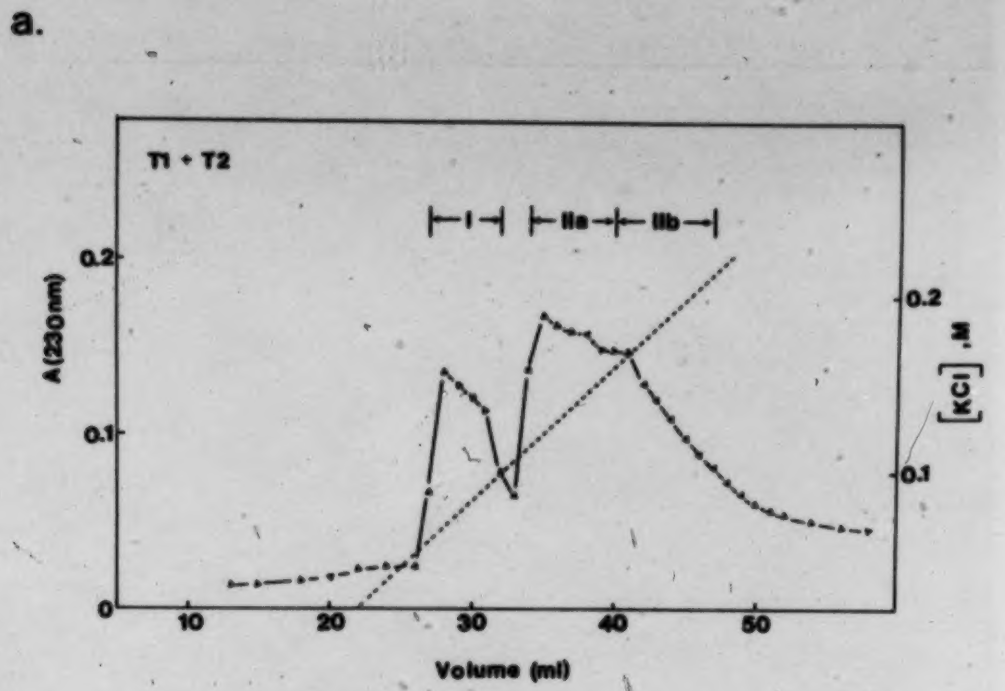

a.
T₃ + CNIB
I
II
A230
0.20
0.10
0
10
20
30
40
Volume (ml)
[KCl], M
0.20
0.10
0
b.
I
II

Fig. 49a. Elution profile of mixture of T1 and T2 (0.5 mg each) on a TnT-Sepharose affinity chromatography column. Same experimental conditions were used as in Figure 47. Fractions were pooled as indicated.

Fig. 49b. SDS-urea polyacrylamide gel of the fractions I, IIA and IIB.


a.
T1 + T2
I
IIa
IIb
A(230nm)
0.2
0.1
0
10
20
30
40
50
Volume (ml)
[KCl] ,M
0.2
0.1

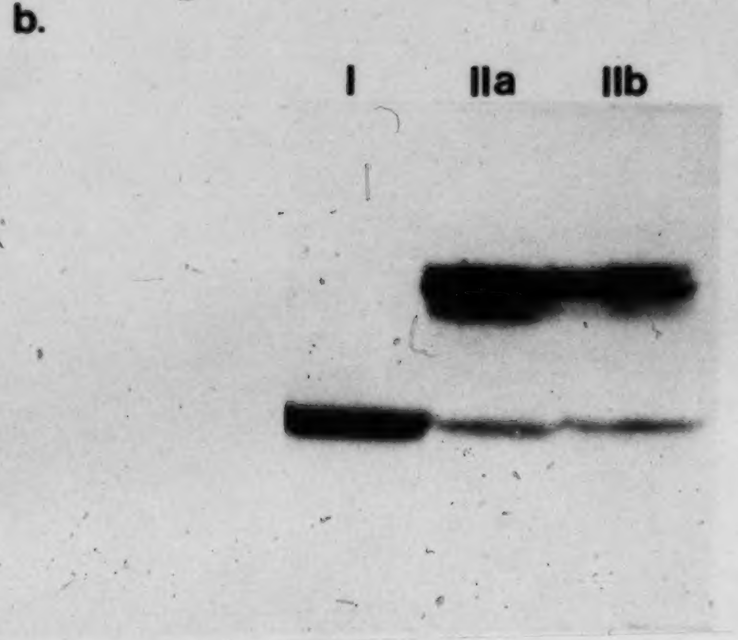

b.
I
IIa
IIb

TABLE Xa

Binding of Mixtures of Fragments to Immobilized TnT

	Peak I		Peak II	
	Elution concentration (M)	Composition[a]	Elution concentration (M)	Composition[a]
T1 + T3	0.05	T1 + T3		
Class I:				
T1 + T2	0.06	T1	0.13	T1 + T2
Cyl + T2	0.05	Cyl	0.14	Cyl + T2
CT1 + T2	0.06	CT1	0.15	CT1 + T2
T1 + CN1B	0.05	T1	0.12	T1 + CN1B
T1 + CT2	0.05	T1	0.15	T1 + CT2
Class II:				
CN1A + CT2	0.05	CN1A	0.11	CT2
T3 + CN1B	0.06	T3	0.11	CN1B

[a] Determined by SDS-urea polyacrylamide gel electrophoresis.

On the basis of the ionic strength at which the second peak was eluted and the composition of this peak, the set of data can be divided into two classes: (1) those in which the second peak was composed of the two components and was eluted at salt concentrations higher than 0.11M and (2) those in which the second peak consisted of one component, which was dissociated from the ligand at the ionic strength required to elute the COOH-terminal fragments. The presence of the two components on the same peak can be explained by an interaction between the two fragments forming a complex which interacted with the immobilized TnT.

Figures 48 and 49 are representative elution profiles of the two classes described. The peaks which emerged from the chromatography of a mixture of T3 and CN1B were symmetrical while those of a mixture of T1 and T2 were nonsymmetrical and broad particularly the second peak. If T1 and T2 were indeed interacting, the mixture of these fragments would contain free T1 and T2 in equilibrium with the complex. T1 and T2 would be eluted at 0.05M and 0.10M KCl respectively, while the complex would be eluted at salt concentrations higher than 0.10M if interaction between the fragments enhances TnT binding. The electrophoretic pattern (Fig. 49b) of the material from the early part of the second peak, IIa, revealed two bands corresponding to T1 and T2 but T2 was of greater intensity, while the later part of the peak, IIb, contained both T1 and T2 in almost equal amounts. These results would indicate that the front of the second peak is due to T2 and the latter part is due to the complex. Thus, the second peak is a composite of the peaks of T2 and the complex and is reflected by its assymetry and broadness. By extrapolation of this interpretation to the other mixtures, the results from this set of experiments can be presented more correctly in Table Xb.

Although the salt concentrations required for the elution of complexes of NH_2-terminal and COOH-terminal fragments were in some cases only marginally higher than that required for the elution of COOH-terminal fragments alone, these experiments have been reproduced more than once and with two separate affinity columns prepared at different times. Thus, we believe that these results are meaningful and significant.

On closer scrutiny, the mixtures of fragments which belong to the first class were composed of polypeptides with the NH_2- and COOH-terminal ends of the parent molecule, except for CN1B, suggesting that the specific interaction between them could be head to tail aggregation. Model building (McLachlan & Stewart, 1975) and chemical modification studies (Johnson & Smillie, 1977) showed that the head to tail polymerization of Tm involved some interactions between basic and acidic residues from the N- and C-termini of the molecule, respectively, as well as hydrophobic interaction of non polar residues, particularly Met-281. The ability of αTm to polymerize is drastically reduced upon the removal of Met-281 by carboxypeptidase A.

TABLE Xb

Binding of Mixtures of Fragments to Immobilized TnT

	Fragments eluted at KCl concentration of		
	0.05 - 0.06M	0.10 - 0.11M	>0.11M
T1 + T3	T1, T3		
Class I:			
T1 + T2	T1	T2	T1 + T2
Cy1 + T2	Cy1	T2	Cy1 + T2
CT1 + T2	CT1	T2	CT1 + T2
T1 + CN1B	T1	CN1B	T1 + CN1B
T1 + CT2	T1	CT2	T1 + CT2
Class II:			
CN1A + CT2	CN1A	CT2	
T3 + CN1B	T3	CN1B	

The observation that CN1B formed a complex with the NH_2-terminal fragments implies that modification of this amino acid to homoserine by CNBr treatment does not entirely eliminate the interactions involved in the head to tail aggregation.

Non-specific interactions between these fragments were ruled out by the lack of interaction between NH_2- and COOH-terminal fragments incapable of head to tail aggregation (as in the second class) as well as by the elution of a mixture of NH_2-terminal fragments T1 and T3 as a single peak at 0.05M KCl.

The observation that binding of fragments to TnT-Sepharose is increased when head to tail aggregation is possible supports the hypothesis that the degree of polymerization of Tm affects the binding affinity of Tm for Tn.

On the other hand, this observation also raises the possibility of Tn binding near or at the end to end overlap. The interaction studies between αTm and its fragments with Tn and its components performed in this study yielded experimental evidence that support the latter possibility as well as evidence that appears to be inconsistent with it.

The evidence which is consistent with this possibility is (1) the COOH-terminal fragments which extends to, or almost to, the C-terminus of the molecule bind to immobilized TnT, Tn and CB1; (2) the complexes of NH_2- and COOH-terminal fragments capable of end to end aggregation exhibit higher binding affinity to immobilized TnT; (3) binding of T1 and T2, which contain the N- and C-termini of αTm respectively, to TnCT and CB1 were well demonstrated by ultracentrifugation, gel filtration and polyacrylamide gel electrophoresis. These results were initially considered as non-specific, but in view of later developments, they may be meaningful and significant. Interaction of NH$_2$-terminal fragments to Tn may be weaker than those of COOH-terminal fragments such that it may be difficult to differentiate whether the binding of T1 to immobilized TnT is specific or non-specific.

If Tn binds to the terminal ends of Tm, then Cy1 and CT1, which have the intact N-terminus of αTm, and T4 and CN1B, which extend to, or almost to, the COOH-terminal end of the molecule, should have exhibited binding when studied by gel filtration and polyacrylamide gel electrophoresis, but they did not. In search for an explanation for this observation, it was noted that all these fragments, Cy1, CT1, T4, CN1B and CN1A were exposed to 8M urea at pH 7.5 in their preparation while the rest of the fragments were not. It is well documented that under these conditions, carbamylation of the free NH$_2$-group occurs (Hirs, 1967). Thus, the modification of the ΣNH$_2$- groups of the lysine residues which abound at the N-terminus of αTm and also distributed throughout the molecule may account for the lack of apparent binding of CT1 and Cy1 to Tn and its components by all the techniques used. This is substantiated by the observation that the uncleaved αTm obtained from the preparation of Cyl (and thus subjected to identical conditions) bound to immobilized Tn but was eluted at appreciably lower ionic strength (0.14) than native αTm.

T4 and CN1B, however, bound to immobilized TnT but not to CB1 or TnCT as shown by gel filtration and polyacrylamide gel electrophoresis studies. This difference may reflect the events occurring during the assay. If we assume that initially the CB1 or TnCT and the fragments were in equilibrium with their complex, this equilibrium is disturbed as the components are separated from the complex and will favor its dissociation especially if the binding has been weakened by carbamylation of the lysyl amino groups. In affinity chromatography, there is always excess of one of the components of the complex which may have the effect of enhancing binding. Interaction studies using gel filtration and polyacrylamide gel electrophoresis were done in 0.15M KCl which may also account for the different results obtained. In any event, these results do not entirely contradict the possibility that the Tn binding site is at the end to end overlap.

2. Other Tropomyosins

Modified rabbit skeletal Tm and tropomyosins from other sources were tested for their binding capacity to immobilized rabbit skeletal Tn and TnT. The ionic strength of the eluent required to dissociate the tropomyosins from the ligands varied (Table XI). Deductions on the nature

TABLE XI

Binding of Different Tropomyosins to Immobilized TnT, Tn and CBl

	Elution [KCl] M		
	TnT-Sepharose	CBl-Sepharose	Tn-Sepharose
Rabbit skeletal Tm	0.20		
αTm	0.20	0.20	0.20
βTm[a]	0.16		0.16
CM-αTm	0.20		
CM-βTm	0.15		
oxidized αTm[b]	0.20		
Cardiac Tm	0.20	0.20	
Horse blood Platelet Tm[c]	0.05	0.04	0.08
Chicken gizzard Tm[d]	0.10		0.11
Frog Tm[e]	0.19		0.19

Protein was kindly provided by

a Dr. Alan Mak
b Dr. Joan Ng
c Mr. Graham Coté
d Dr. William Lewis
e Dr. Michael Barany

and localization of the binding site of Tn on Tm may be made from these observations.

The same observed affinities of both carboxymethylated Tm and oxidized Tm as that of the reduced form reflect the lack of involvement of the cysteine residue of Tm in the interaction. βTm bound to Tn and TnT to a slightly lesser extent than αTm although the sequence around cysteine-190, the putative Tn binding site, is very similar to that of αTm (Fig. 50). The only drastic replacement in βTm is the substitution of serine, residue 229, and histidine, residue 276, in αTm with glutamic acid and asparagine, respectively (Mak & Smillie, unpublished). Both changes, which may be responsible for the reduced affinity of βTm for Tn, occur beyond the predicted binding region and nearer the end of the molecule.

On the other hand, horse blood platelet Tm exhibited the same binding behavior as the fragments derived from the NH_2-terminal half of αTm. The lack of interaction of this protein with Tn and TnT was unexpected in view of the similarity of the sequence of rabbit skeletal αTm and horse blood platelet Tm at the region near cysteine 190 (Fig. 50). A charge replacement at residue 216 (glutamic acid for glutamine) has been found (Coté, et al., unpublished). Unfortunately, there is no information on the sequence of chicken gizzard Tm, which does not bind more tightly than the COOH-terminal fragments of αTm.

The evidence obtained from this set of experiments

Fig. 50. Partial amino acid sequences rabbit skeletal αTm (first line) [Stone, _et al_., (1974)], βTm (second line) [Mak & Smillie, unpublished], and horse blood platelet Tm (third line) [Coté, et al., unpublished].

- denotes no replacement.

	183 Ala	184 Glu	185 Leu	186 Ser	187 Glu	188 Gly	189 Lys	190 Cys	191 Ala	192 Glu	193 Leu	194 Glu
Rabbit Skeletal αTm												
βTm	-	-	Val	Ala	-	Ser	-	-	Gly	Asp	-	-
Horse blood Platelet Tm	-	-	Val	-	-	Leu	-	-	Gly	Asp	-	-

	195 Glu	196 Glu	197 Leu	198 Lys	199 Thr	200 Val	201 Thr	202 Asn	203 Asn	204 Leu
	-	-	-	-	Ile	-	-	-	-	-
	-	-	-	-	Asn	-	-	-	-	-

	205 Lys	206 Ser	207 Leu	208 Glu	209 Ala	210 Gln	211 Ala	212 Glu	213 Lys	214 Tyr	215 Ser	216 Gln	217 Lys
	-	-	-	-	-	-	-	Asp	-	-	-	Thr	-
	-	-	-	-	-	Ala	Ser	Glu	-	-	-	Glu	-

	218 Glu	219 Asp	220 Lys	221 Tyr	222 Glu	223 Glu	224 Glu	225 Ile	226 Lys	227 Val	228 Leu	229 Ser
	-	-	-	-	-	-	-	-	-	Leu	-	Glu
	-	-	-	-	-	-	-	-	-	Leu	-	-

	230 Asp	231 Lys	232 Leu	233 Lys	234 Glu	235 Ala	236 Glu	237 Thr	238 Arg
	Glu	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-

suggests that consideration must be given to the possibility that the site of Tn binding may be located at or close to the C-terminus of the Tm molecule and may even involve the NH_2-terminal and COOH-terminal overlap regions of Tm aggregation.

B. INTERACTIONS WITH F-ACTIN

1. Rabbit Skeletal αTm and its Fragments

The interaction between αTm and F-actin was investigated by the cosedimentation technique. The presence of both proteins in the pellet, obtained from centrifugation of a mixture of these proteins and analyzed by SDS-polyacrylamide gel electrophoresis, indicated complex formation. The reliability of the technique was tested by determining the ratio of αTm to actin in the pellet. Standard curves of the proteins with respect to their Coomassie blue staining properties were constructed (Fig. 51) since Potter (1974) has reported that equal amounts of Tm and actin stain to different extents. The procedure used was found satisfactory since the ratio calculated for the proteins in the pellet was one mole Tm to seven moles actin which is the same as that found in the myofibril (Potter, 1974).

With the exception of non-polymerizable Tm, none of the fragments tested, T1, T2, T3 and Cy1, bound to F-actin (Fig. 52). Since these fragments came from representative regions of the molecule, it was assumed that all the

Fig. 51. Dependence of densitometric peak areas on myofibrillar protein concentration. Various amounts of actin (o) and αTm (Δ) were applied on SDS-polyacrylamide disc gels. The Coomassie Blue stained gels were scanned at 590 nm.

9

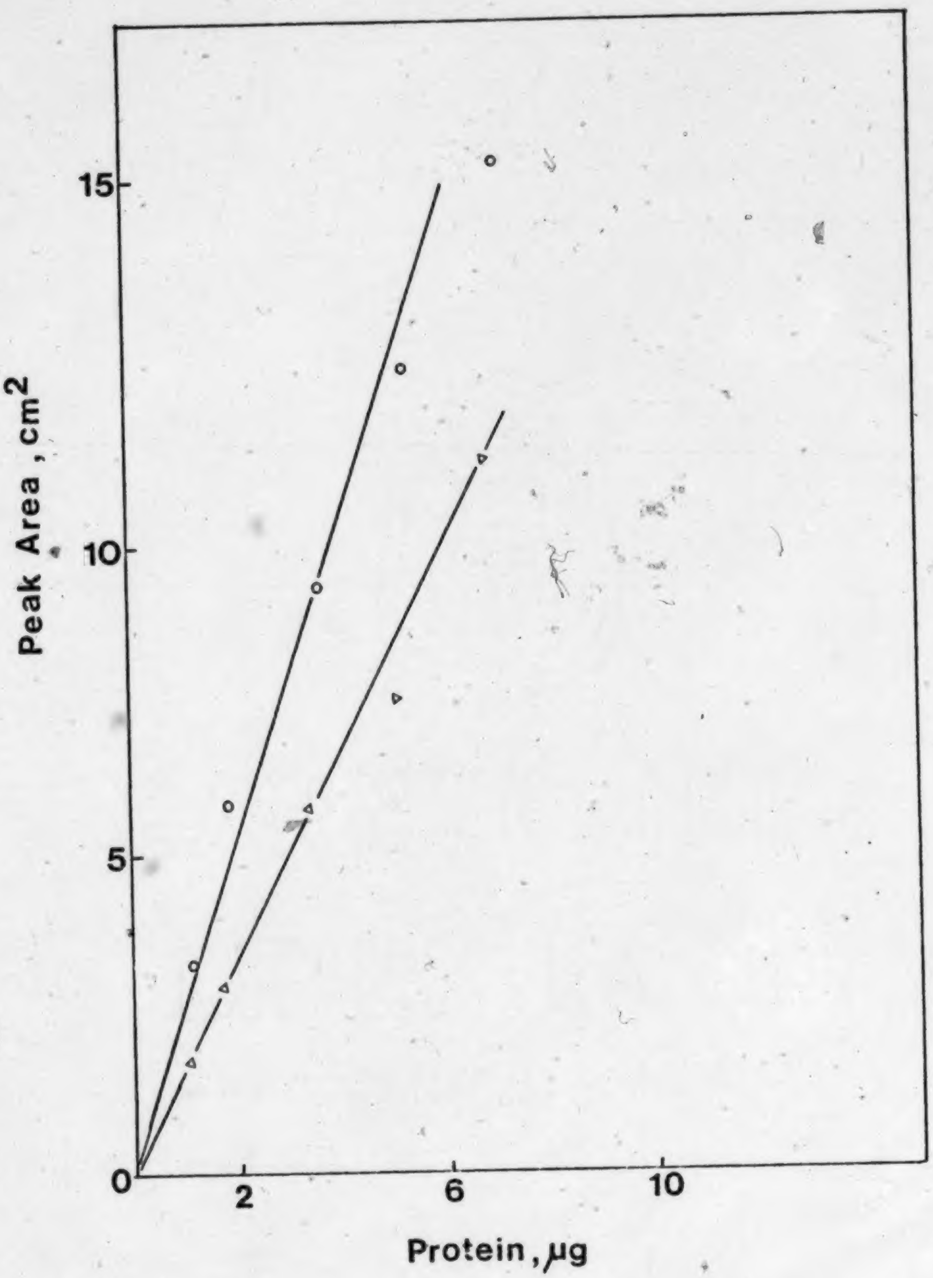
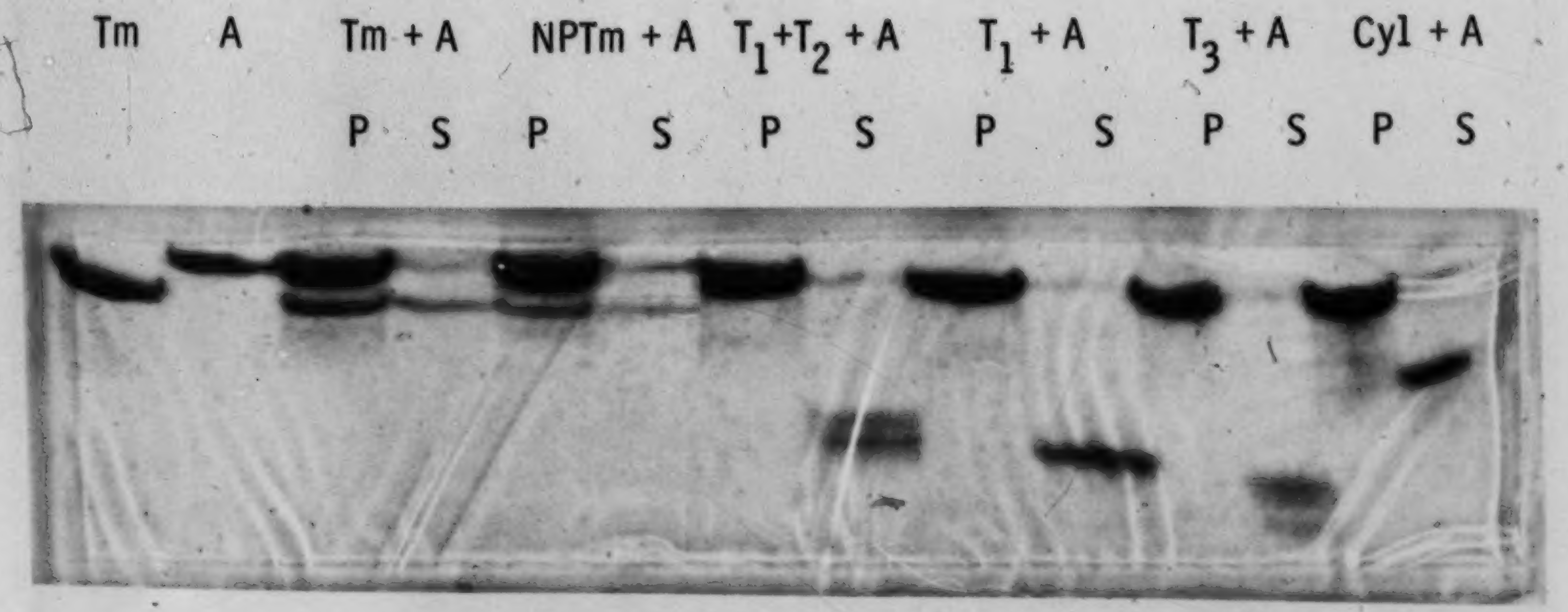


Fig. 52. Determination of interactions of F-actin (A) and αTm or its fragments by cosedimentation method. Mixtures of F-actin (2 mg) and αTm or its fragments (0.4 mg) in 30 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 2 mM ATP, 1 mM imidazole, pH 7.0, were incubated for 2 hours at room temperature and centrifuged at 20,000 g at 4°C for 15-20 hours. The material obtained from the supernatant (S) and pellet (P) were examined in SDS-polyacrylamide gel.


Tm
A
Tm + A
NPTm + A
T_1+T_2 + A
T_1 + A
T_3 + A
Cyl + A
P
S
P
S
P
S
P
S
P
S
P
S

A = actin; P = pellet; S = supernatant.

other fragments isolated would also exhibit the same property. Addition of large excess of the fragments or performing the assay in the presence of Tn did not alter the result.

Theoretically Tm is expected to have seven or fourteen binding sites for actin, evenly distributed throughout the molecule. Thus each of the fragments tested should have two or more actin binding regions. The lack of interaction displayed by all of the fragments implies that the strength of interaction between actin and the individual actin binding sites on Tm is relatively weak. Only in the case where one has the additive effect of all the sites is the strength of binding high enough to be detected by the methods employed in this work. It is not surprising that carboxypeptidase-treated Tm (i.e. non-polymerizable Tm) was found to cosediment with F-actin since its actin binding sites would be left essentially intact by this minor modification in its structure.

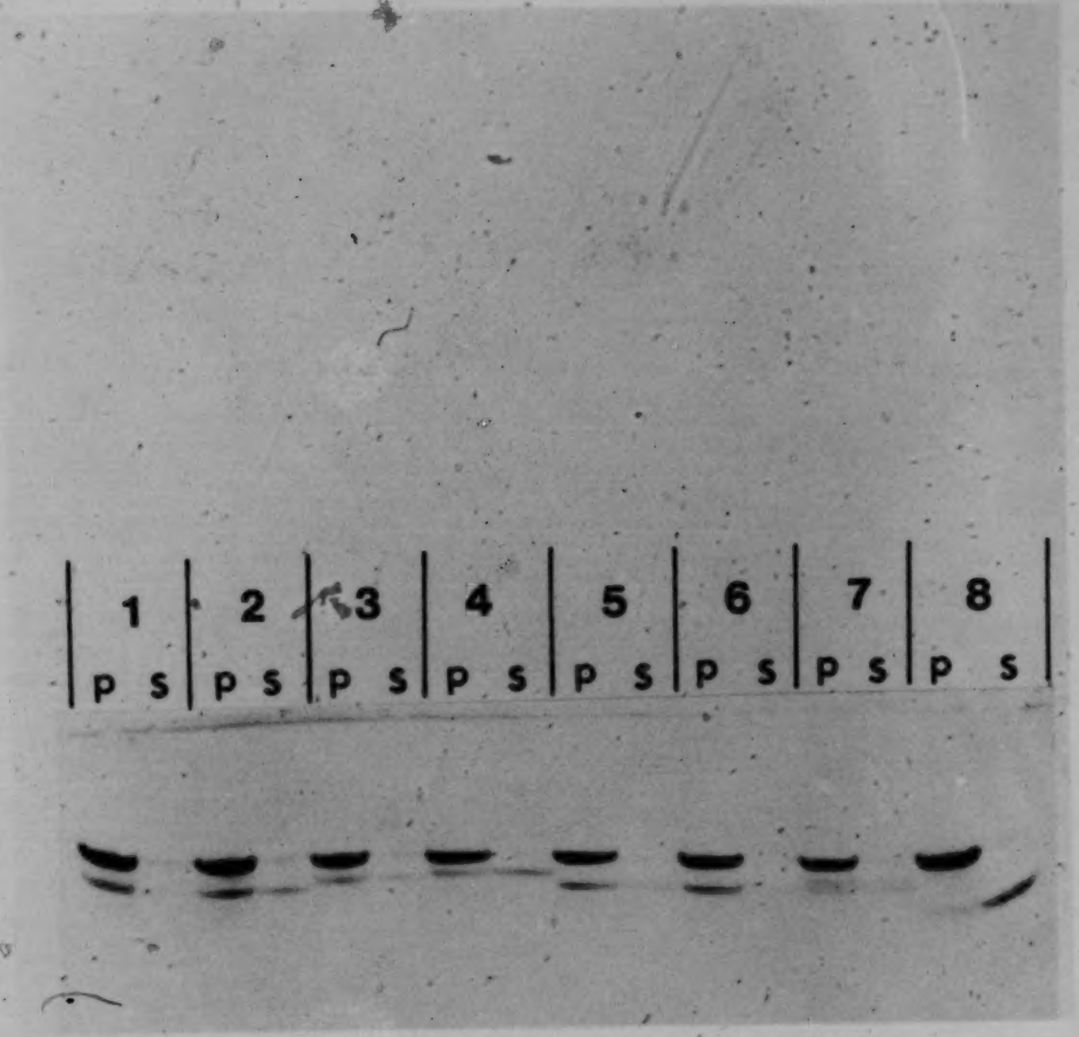
2. Other Tropomyosins

Using the same technique, the binding of rabbit skeletal F-actin to different types of tropomyosins, rabbit skeletal Tm, αTm, carboxymethylated α and βTm, rabbit cardiac Tm, frog Tm, chicken gizzard Tm and horse blood platelet Tm, was investigated. All the different tropomyosins tested bound to F-actin but some to a lesser extent than others (Fig. 53). Unfractionated rabbit skeletal Tm bound to about the same extent as rabbit αTm, rabbit cardiac

Fig. 53. Determination of interactions of F-actin and other tropomyosins by cosedimentation method. Same conditions were used as in Figure 52. The materials obtained from the precipitate (P) and supernatant (S) of mixtures of F-actin and other tropomyosins

(1) unfractionated rabbit skeletal Tm
(2) rabbit skeletal αTm
(3) carboxymethylated αTm
(4) carboxymethylated βTm
(5) cardiac Tm
(6) frog Tm
(7) chicken gizzard Tm and
(8) horse blood platelet Tm

were examined in SDS polyacrylamide gel.


1
2
3
4
5
6
7
8
P S
P S
P S
P S
P S
P S
P S
P S

Tm and frog Tm. Neither chicken gizzard Tm nor horse blood platelet Tm bound to F-actin as tightly as rabbit skeletal Tm. These observations may reflect the type of actin present in the various sources from which these proteins have been extracted. These results imply that the actin from the striated muscle appear to be similar but are different from the actin present in the smooth muscle of the chicken gizzard and the non-muscle cell, horse blood platelet.

CHAPTER VI

CONCLUDING DISCUSSION

Tropomyosin is almost completely α helical below 25°C in the absence of denaturants. However, denaturation studies on αTm have indicated the presence of regions of less helical structure. In this study, some of these regions were localized by brief and prolonged exposure to the proteolytic enzymes, trypsin and chymotrypsin. Since Tm contains a high proportion of basic and bulky hydrophobic amino acids, the preferential sites of cleavage of these enzymes have been taken as an indication of less ordered regions.

Digestion of αTm with trypsin at 0°C for one minute at an enzyme to substrate ratio of 1:200 caused a rapid cleavage of peptide bond 133-134. This single point cleavage implied that this bond is in a region which is least helical in the αTm molecule. The local disruption of the coiled coil structure in this region may be accounted for by the presence of Asp-137 in a hydrophobic core position. Unlike other polar amino acids in these positions, the side chain of aspartic acid is not sufficiently long to direct its charged moiety towards the exterior of the hydrophobic core. Prolonged exposure to the enzyme led to hydrolysis of portions of T1 (1-133) and T2 (134-284) producing T3 (13-125), T4 (183-284) and T5 (183-244). These fragments were resistant to further action of the enzyme reflecting the extent of order of these regions of the molecule.

On the other hand, chymotryptic digestion of αTm for 5 minutes at 25°C at an enzyme to substrate ratio of 1:500 produced fragments CT1 (1-169) and CT2 (170-284) from the cleavage of peptide bond 169-170. The reason for the disruption of the coiled coil structure around this bond is not as obvious as that around Asp-137. The presence of a disruption of lesser extent in this region could account for the more drastic conditions required to accomplish the cleavage than those for trypsin. An additional factor that could have favored the cleavage at Ile-169 is its position at a hydrophobic part of the sequence, the only four consecutive bulky hydrophobic residues in αTm. The susceptibility of this type of sequence was also implied by the failure of the enzyme to hydrolyze peptide bonds Ile-130 and 143 which are in the vicinity of Asp-137 and thus located in a presumably less helical region but surrounded by charged residues.

Conformational analysis of the sequence of αTm done in search for regions of low helical contents, did not show any correlation with the sites of cleavage by trypsin and chymotrypsin. However, the conclusion made from this analysis, that the termini of the molecule are the regions which are least ordered, may not have been evident from the results of enzymic digestion. The reason for this is that these experiments were performed at low ionic strengths, under which conditions the ends of the molecule are involved in head to tail aggregation. This interaction could have made the ends of αTm resistant to the initial action of the enzymes.

The presence of a limited number of certain amino acids in the molecule allowed the preparation of large fragments of αTm by specific chemical cleavages. Cy1 (1-189) and Cy2 (190-284) were produced by cleavage of the peptide bond on the NH_2-terminal side of Cys-190 by the combined action of DTNB and KCN. CNBr digestion of αTm at the methionine residues produced CN1A (11-127) and CN1B (142-281).

Investigation of the properties of all the fragments isolated, by circular dichroism, revealed that the NH_2-terminal half of the molecule is more helical and stable to heat denaturation than the COOH-terminal region. Lehrer also observed the instability of the region around Cys-190 and that introduction of the disulfide cross-link in the molecule caused a local disruption of the α helix near physiological temperature. The biological significance of this property of αTm is not clear but it may have a correlation with the proposed location of the Tn binding site.

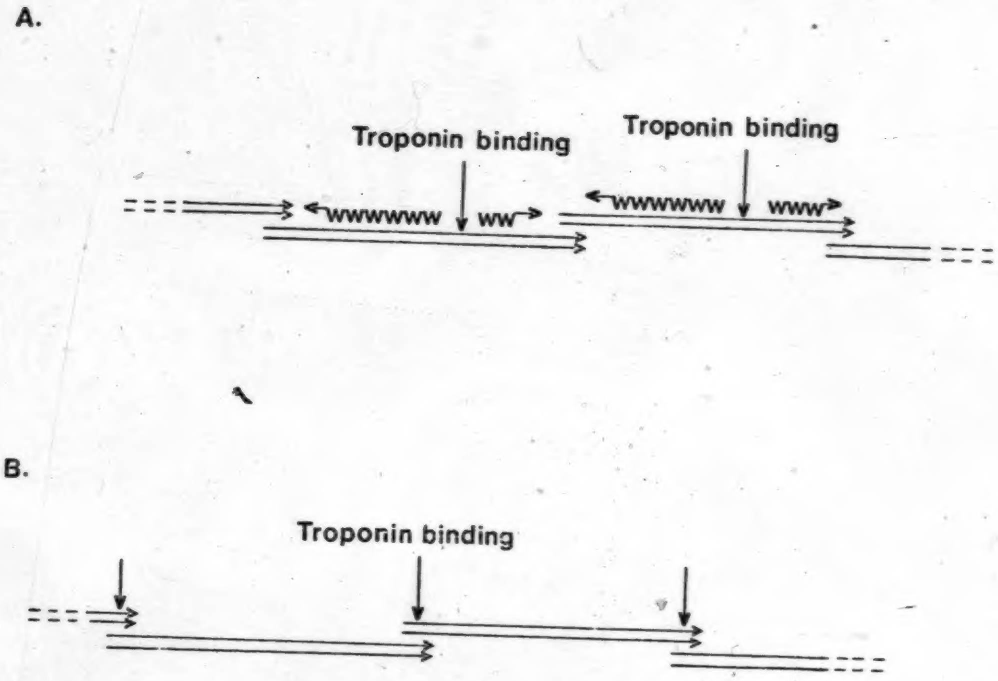
Interactions of the fragments of αTm with Tn and its components were investigated using several techniques, ultracentrifugation, polyacrylamide gel electrophoresis, gel filtration and affinity chromatography. Binding studies with immobilized TnT showed the independence of the Tm-TnT interaction on the presence of Ca^{++}, Mg^{++} and reducing agent, which implied no involvement of the cysteine residue in the interaction. All the NH_2-terminal fragments bound to the immobilized TnT with the same affinity as those of other helical proteins and thus, was considered non-specific. The COOH-terminal fragments bound more tightly than the NH_2-terminal fragments but less tightly than intact αTm. Similar observations were obtained from studies with immobilized Tn and CB1. These results are consistent with the hypothesis that the Tn interaction site on Tm is about one-third of the distance from the C-terminus of the molecule.

Non-polymerizable Tm which is only about 8 residues shorter on the COOH-terminal end of αTm, exhibited binding to the same extent as the COOH-terminal fragments. This observation could be accounted for by the multi-binding sites of Tm polymer but also raises the possibility that there might be a correlation between the strength of Tm-Tn interaction and the extent of polymerization. This is also suggested by the increase of the viscosity of αTm on addition of Tn. This possibility was substantiated by results from binding studies on mixtures of fragments of αTm with immobilized TnT. Formation of complexes between fragments capable of head to tail aggregation was indicated by these experiments. These complexes exhibited tighter binding to immobilized TnT.

On the other hand, these results may also indicate that the Tn binding site is very close to the COOH-terminal end of the molecule or at the end to end overlap. This latter possibility is consistent with the findings that horse blood platelet Tm does not bind to TnT inspite of the similarity of its sequence to that of αTm at the putative Tn binding site. Its lack of binding to TnT may be accounted for by its different NH_2- and COOH-terminal sequences from those of αTm.

The binding studies using the other techniques, ultracentrifugation, gel filtration and polyacrylamide gel electrophoresis revealed that T1 and T2 which have the N- and C-termini of αTm respectively, bound to TnCT and CB1. Since none of the other fragments tested bound to TnCT and CB1, these interactions were initially considered as non-specific but in view of the later developments, they may be significant. The lack of binding of Cy1 and CT1 which have the N-terminus of αTm and T4 which contains the C-terminus of the molecule may be due to carbamylation of the ΣNH_2-group of their lysine residues during their preparation. These fragments were exposed to 8M urea at pH 7.5 while T1 and T2 were not.

Thus, two possibilities for the location of the Tn binding site are compatible with the results obtained from this study. The first possibility (Fig. 54a) locates the Tn interaction site one third from the C-terminus of the molecule. Binding of Tn to Tm would induce a conformational change which is transmitted to the ends of the molecule.


A.
Troponin binding
Troponin binding
wwwwww
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www
B.
Troponin binding

Fig. 54. Two possibilities for the location of the Tn interaction site on αTm.

a. Tn can bind at a region one-third of the distance from the c-terminus of the molecule. This interaction causes a conformational change which is transmitted to the ends of the molecule.

b. Tn can bind to the end to end overlap of the αTm molecules.

This would then enhance head to tail aggregation and vice versa. The second possibility (Fig. 54b) locates the Tn binding site at the end to end overlap and the strength of interaction between the two proteins is also influenced by the degree of head to tail polymerization and vice versa. Differentiation between these two possibilities is not possible with the present evidence and thus, awaits further work in this aspect.

The inconsistency of this latter possibility with the conclusion, obtained from the study of ordered aggregates of Tm, may arise from the conditions used in these experiments. The crystals of Tm were prepared at low pH and the magnesium paracrystals were prepared at high Mg^{++} concentration, both of which are non-physiological conditions. But if these aggregates are indeed representative of the physiological structure, interaction of Tn with some regions (like the ends of the molecule) of αTm in this form could be inhibited by steric effects. It is interesting to note that αTm is phosphorylated at a serine residue very close to C-terminus of the molecule, Ser-281. This charged group may be involved in the end to end interaction but it may also be involved in the Tm-Tn interaction if the Tn binding site is at the overlap.

None of the fragments bound to F-actin except for non-polymerizable Tm. This suggested that the strength of interaction between actin and individual actin binding site on Tm is relatively weak and that the additive effect of all the sites are required for binding. Tm from different sources bound F-actin to different extents, reflecting the difference of the actin found in various sources.

Thus the results of this study suggest that Tm should not be considered strictly as a rigid rod but rather as a flexible rod with regions of varying stability. Under physiological conditions, an equilibrium may exist between the melted and unmelted states of these regions. Since these unstable regions occur at the COOH-terminal part of the molecule, where Tn binding has been implicated, they may be involved in the movement of Tm into the groove of the actin helix when TnC binds Ca^{++}.

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